Systematics and mating systems of two fungal pathogens of opium poppy: the heterothallic *Crivellia papaveracea* with a *Brachycladium penicillatum* asexual state and a homothallic species with a *Brachycladium papaveris* asexual state

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Abstract: This paper presents a systematic revision of the fungal opium poppy (*Papaver somniferum* L.) pathogens formerly known as *Pleospora papaveracea* (de Not.) Sacc., along with allied asexual states formerly placed in *Dendryphion*. The revision is based on analysis of phylogenetic relationships, comparative morphology, and analysis of mating systems. Using morphology, 18S and ITS rDNA, we established that these species belong to the *Alternaria* group rather than to *Pleospora*, a conclusion supported by the Shimodaira–Hasegawa test. For these fungi, we erect the new genus *Crivellia*, with *Crivellia papaveracea* as type. ITS rDNA analyses suggested with moderate support *Alternaria brassicicola* (Schw.) Wiltshire, *Alternaria japonica* Yoshii, and *Ulocladium alternariae* (Cooke) Simmons as *Crivellia*'s closest relatives. Combined ITS, partial *GPD* and *EF-1 alpha* analyses confirmed earlier studies that show that asexual isolates in the *Crivellia* lineage of poppy pathogens represent two closely related species. Because *Dendryphion* was determined to be polyphyletic, the former genus *Brachycladium* was resurrected for *B. penicillatum* Corda and *B. papaveris* (K. Sawada) Shoemaker & Inderbitzin, the *Crivellia* asexual states that had been in *Dendryphion*. Molecular and morphological comparison with isolates from field-collected ascomata and morphological comparison with the type specimen of *P. papaveracea* indicated that *B. penicillatum*, and not *B. papaveris*, is the anamorph of *C. papaveracea*. The mycelia from single conidium or single ascospore isolates, including mycelia from 14 single ascospores from one field-collected *C. papaveracea* ascoma, either have a *MAT1-1* or *MAT1-2* gene and are thus heterothallic. In contrast, each single-conidium isolate of *B. papaveris* has an incomplete *MAT1-2* gene fused to a *MAT1-1* region and is inferred to be homothallic. We speculate that ancestral *MAT* fusion might have led to speciation in *Crivellia*.

Key words: life history evolution, mating system evolution, sympatric speciation, *MAT* fusion, *Papaver somniferum*, *Dendryphion nanum*.

Résumé : On passe en revue la systématique des champignons pathogènes du pavot à opium (*Papaver somniferum* L.) connus jusqu'ici comme le *Pleospora papaveracea* (de Not.) Sacc., en incluant les stades imparfaits auparavant placés dans le genre *Dendryphion*. La révision est basée sur l'analyse des relations phylogénétiques, de la morphologie comparée et de l'analyse des systèmes de croisement. En utilisant la morphologie et les données des 18S et ITS du rADN, les auteurs ont établi que ces espèces appartiennent au groupe *Alternaria* plutôt qu'au groupe *Pleospora*, une conclusion qui est supportée par le test Shimodaira–Hasegawa. Pour ces champignons, les auteurs ont constitué le nouveau genre *Crivella*, le type étant *Crivella papaveracea.* Des analyses de l'ITS du rADN suggèrent comme plus proches parents du *Crivella,* les *A. brassicola, A. japonica* et *Ulocladium alternariae*. Une combinaison des analyses ITS, *GPD* partiel et *EF-1 alpha* confirme des études antécédentes montrant que des isolats asexués de la lignée des *Crivella* pathogènes du pavot représentent deux espèces étroitement apparentées. Après avoir déterminé que le *Dendryphion* est polyphylétique, on a ressuscité l'ancien genre *Bradycladium* pour accommoder les *B. penicillatum* Corda et *B. papaveris* (K. Sawada), stades sexuels du *Crivella* qui avaient été attribués aux *Dendrophion*. Une comparaison moléculaire et morphologique avec des isolats obte-

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nus d'ascomata recueillis aux champs, et une comparaison morphologique avec le spécimen type du *P. papaveracea* indiquent que le *B. penicillatum,* et non pas le *B. papaveris,* constitue l'anamorphe du *C. papaveracea*. Des mycéliums issus de spores ou de conidies isolées, incluant des mycéliums provenant de 14 spores individuelles obtenues d'un ascoma du *C. papaveracea* récolté sur le terrain, possèdent le gène *MAT1-1* ou *MAT1-2* et sont ainsi hétérothalliques. À l'opposé, chaque isolat mono-conidial du *B. papaveris* possède un gène *MAT1-2* incomplet fusionné à la région *MAT1-1,* et on en déduit qu'il est homothallique. Les auteurs pensent qu'une fusion ancestrale de MATs pourrait avoir conduit à la spéciation du genre *Crivella*.

Mots clés : évolution du cycle vital, évolution du système de croisement, spéciation sympatrique, fusion de *MAT*, *Papaver somniferum*, *Dendryphion nanum.*

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Introduction

The opium poppy, *Papaver somniferum* L., is an ancient agricultural crop that originated in the Mediterranean region (Braun 1905; Krzymanski and Jönsson 1989). It is best known for its alkaloid-rich latex, the base of opium and heroin. The seeds, however, are nontoxic, and have been used as food, feed, and for several other purposes around the world (Duke 1973; Veselovskaia 1976).

Various studies have been dedicated to the cultivation of this plant and its associated pathogens (Flachs 1936; Pape 1944). The fungal species that have been included under *Pleospora papaveracea* (de Not.) Sacc. frequently cause large crop losses (Christoff 1931; Reinmuth 1942; Darpoux 1945; Grümmer 1952; Milatovic 1952; Radulescu and Perseca 1964). This destructive potential has led to research on the fungi as biocontrol agents to limit poppy cultivation (Bailey et al. 2000; Del Serrone and Annesi 1989; O'Neill et al. 2000).

Despite the obvious importance of taxonomy of pathogens, the systematics of *P. papaveracea* has been so uncertain that it has impeded the comparison and integration of pathological and physiological data accumulated during almost a century of scientific studies of fungal opium poppy pathogens. At the genus level, *P. papaveracea* has been recognized as an anomalous species of *Pleospora*. Müller (1951) included *Cucurbitaria papaveracea* de Not. as a synonym of *Pleospora calvescens* (Fr.) Tul., which he said is "eine isoliert stehende Art der Gattung." Wehmeyer (1961, p. 39) proposed the section *Leptosphaerioides* typified by *Pleospora pellita* (Fr.) Rab. and segregated it from *Pleospora herbarum* (Fr.) Rab., the type species of *Pleospora*. Shoemaker (1968) noted that Wehmeyer's concept of *Pleospora pellita* was broad and included *P. papaveracea* among others. Wehmeyer's segregration indicated that he did not consider *P. pellita* (or by extension, *P. papavaeracea*) closely allied to the type of the genus. Crivelli (1983, p. 51) suggested possible affinity with *Pyrenophora* and, for the anamorph, *Drechslera*.

Aware of the taxonomic problems, Shoemaker (1968) used morphological analyses of various collections including type material with the goal of finding the correct names for the sexual and asexual states. He concluded that *Pleospora papaveracea* contained one sexual species with the asexual state *Dendryphion penicillatum* (Corda) Fr. Crivelli (1983) agreed with this conclusion. Applying molecular tools, Farr et al. (2000) investigated the genetic diversity of isolates identified as *Pleospora papaveracea* with AFLP markers. Contrary to Shoemaker's studies, they found two species among the isolates, one of which formed a sexual state whereas the other did not. The AFLP variation correlated with morphological features of the asexual states as well as formation of ascomata in pure culture. The asexual isolates referred to as *Dendryphiella* sp. had large, five to seven-septate conidia. Single-conidium isolates of *Dendryphiella* sp. formed a *Pleospora papaveracea*-type sexual state in culture (Farr et al. 2000), from which we infer that this species is homothallic. The other asexual state, *D. penicillatum* had smaller, mainly three-septate conidia, and no sexual state was obtained in culture. From this evidence and without comparisons to type material, Farr et al. (2000) concluded that the correct asexual state of *Pleospora papaveracea* was *Dendryphiella* sp., and that *D. penicillatum* was a distinct asexual species with no known sexual state.

The results presented in this paper support Farr et al.'s (2000) finding of two species among poppy isolates. Confusingly however, the nomenclature of the anamorphs and teleomorphs required reconsideration. To help keep track of the organisms, the revised nomenclature will be used throughout this text. For the anamorphs, *Brachycladium penicillatum* corresponds to *D. penicillatum*; '*Brachycladium papaveris*', a new combination, corresponds to *Dendryphiella* sp. sensu Farr et al. (2000). The teleomorph of *B. penicillatum*, '*Crivellia papaveracea*' corresponds to *Pleospora papaveracea* sensu Shoemaker. Farr et al. (2000) applied the name *Pleospora papaveracea* to the sexual state of *Dendryphiella* sp., but no specimens of the mature teleomorph of *Dendryphiella* sp. are extant nor are their teleomorph illustrations unequivocally derived from *Dendryphiella* sp. Consequently, the teleomorph cannot be described and named and is referred to in this text as the homothallic *Crivellia* species.

With the goal of creating a stable, natural, and practical taxonomic system for the opium poppy fungi formerly placed in *Pleospora*, we used a combination of molecular and morphological tools. To identify their closest taxonomic relatives, we used phylogenetic analyses of ribosomal 18S and ribosomal internal transcribed spacer region (ITS) DNA sequence data. To study the relationships among the poppy pathogens at the species level we analyzed sequences of the ITS regions and of fragments of two protein-coding genes, the *glyceraldehyde-3-phosphate dehydrogenase* gene (*GPD*) and the *elongation factor-1 alpha* gene (*EF*) for 22 isolates. Among these isolates, we chose cultures originally from

Meffert (1950) and Farr et al. (2000), so that we could correlate our results with data from earlier studies. To evaluate mating systems, we used polymerase chain reaction (PCR) and DNA sequencing of the mating type genes, since in the related *Cochliobolus* arrangements and distribution patterns of the mating type genes correlate with mating systems (Yun et al. 1999). Applications of names were justified through comparisons with literature descriptions and with type specimens.

Materials and methods

Designation of epitype specimens

In addition to morphological studies of type specimens, epitype specimens were selected to serve as interpretative types for mating and molecular studies and permit critical identification under Article 9 in the *International Code of Botanical Nomenclature* (Greuter et al. 2000). Cultures derived from epitype specimens are herein designated "ex epitype".

Fungal strains and sources of sequences

Fresh ascomata of *Crivellia papaveracea* isolate P354 on a decaying stem of *Papaver rhoeas* L. were collected by W. Jaklitsch in Vienna, Austria, and sent to us via Prof. Margaret Barr (Table 1). Drops of ascospore and conidium suspensions in sterile tap water were deposited on tap water agar Petri dishes (Hawksworth et al. 1995). Single germinated ascospores and conidia were picked up by forceps and transferred to V8 agar plates (Hawksworth et al. 1995).

Twenty-three additional strains were obtained from various sources (Table 1). These included two outgroup taxa, *Alternaria japonica* Yoshii and *Ulocladium alternariae* (Cooke) Simmons as well as *Dendryphion nanum* (Nees:Fr.) S. Hughes to assess the monophyly of *Dendryphion*. All cultures were maintained on V8 agar plates. An additional 14 single ascospore isolates from *C. papaveracea* isolate P354 (not shown in Table 1) were used to screen for mating type, and used in crosses on V8 agar plates on a laboratory bench at room temperature in natural and artificial light during the day and in darkness at night.

Two alignments, the 18S rDNA alignment M1158 of genera in the Pleosporales (Inderbitzin et al. 2002), and the ITS alignment M1585 of *Alternaria* and allies (Pryor and Bigelow 2003) were retrieved from TreeBASE. See Inderbitzin et al. (2002) and Pryor and Bigelow (2003) for GenBank accession numbers. Other GenBank sequences used were *Trematosphaeria heterospora* (de Not.) G. Winter (AY016354), *Westerdykella cylindrica* (Malloch & Cain) von Arx (AY016355), *Mycosphaerella mycopappi* A. Funk & Dorworth (U43463), and *Lewia scrophulariae* (Desm.) M. E. Barr & E. G. Simmons (anamorph: *Alternaria conjuncta* E. G. Simmons) (AF392988).

DNA extraction and sequencing

Mycelium was scraped off the surface of a plate, and DNA was extracted using a standard phenol–chloroform extraction method (Lee and Taylor 1990). PCR reactions were performed with ReadyToGo PCR Beads, or puReTaq Ready-To-Go PCR Beads (Amersham Biosciences, Piscataway, New Jersey, USA), following the manufacturer's instructions. DNA sequencing reactions were set up using 4 μL of Big Dye Terminator Cycle Sequencing Kit version 2.0 or 3.0 (Applied Biosystems, Foster City, California, USA), 100–200 ng of PCR product, and 3.2 pmol of sequencing primer per 20 μL final reaction volume. The following regions in the nuclear genome were PCR amplified for the respective isolates: 18S ribosomal DNA region (18S rDNA) of *Crivellia papaveracea* strain P128 using NS1 and cITS5, the complement of ITS5 (White et al. 1990); for isolates in Table 1, the ribosomal internal transcribed spacer regions (ITS) using primers ITS4 and ITS5 (White et al. 1990), parts of the *GPD* gene using forward primer GPD1 (Berbee et al. 1999) and reverse primer GPD3R, and a fragment of the *EF* gene using forward primer EF446f and reverse primer EF3r (Inderbitzin et al. 2005). The following sequencing primers were used: Bas3, cBas3 (Inderbitzin et al. 2001), NS1 and cITS5 for 18S rDNA, ITS5 or internal ITS1 (White et al. 1990), ITS4 or internal ITS871r (Inderbitzin et al. 2005) for the ITS region, GPD1 or internal GPD2033f (Inderbitzin et al. 2005), and internal GPD2 (Berbee et al. 1999) for *GPD*, and internal EF451f or mostly EF462f and EF3r (Inderbitzin et al. 2005) for *EF*.

Isolates of *C. papaveracea* and *B. papaveris* were screened for the presence of mating type genes using mating type specific primers. These were forward primer PaAlphaF (5′-GCT GAG GCG GAG GTC GAC AG-3′) and reverse primer PaAlphaR (5′-GGA GAG CTT CTT CAT GGG CC-3′) amplifying the diagnostic alpha box of the *MAT1-1* gene, and forward primer Jen2F and reverse primer Jen2R (Inderbitzin et al. 2005) on the diagnostic HMG box of the *MAT1-2* gene. PCR products were separated on a 3% agarose gel formulated to resolve small bands, containing one-third SeaKem GTG agarose (Cambrex Bio Science Walkersville, Inc, Walkersville, Maryland, USA) and two-thirds NuSieve GTP agarose (FMC Bioproducts, Rockland, Maine, USA), a low-melting agarose. For verification of the PCR bands obtained in the mating type screening, some PCR bands were chosen for sequencing. *Crivellia papaveracea* isolate P128 was selected for sequencing parts of the *MAT1-1* region using primer ORF556f (Inderbitzin et al. 2005) as forward primer on the ORF1, and PaAlphaR as reverse primer on the alpha box of *MAT1-1*. For *C. papaveracea* isolate P396, parts of the *MAT1-2* gene region were PCR amplified using ORF556f, and reverse primer Jen2R on the HMG box of the *MAT1-2* gene. *Brachycladium papaveris* isolate P390 was chosen for sequencing of the fused mating type gene region. The PCR primers were ORF556f placed in the ORF1, and the reverse primer PaAlphaR on the alpha box of the *MAT1-1* gene. PCR products destined for sequencing were purified by an ethanol precipitation. The *C. papaveracea* isolate P128 partial *MAT1-1* region DNA sequence was determined using primers ORF556f and PaAlphaR, the *C. papaveracea* isolate P396 partial *MAT1-2* region sequence with ORF556f and PaM2f (5′-CTC ACC ATT TCG TTG AAT TAC C-3′) as forward primers, and in reverse with Jen2R and PaM2r (5′-GGT AAT TCA ACG AAA TGG TGA G-3′). The fused mating type gene region of *B. papaveris* isolate P390 was sequenced in the forward direction with ORF556f, PaM2f, PaHMGf (5′-ATG TCA TAG CTA CAC GCT GC-3′), P351f (5′-TGG CGC TCT AGC CTG CTG C-3′), and in reverse direction with PaAl-

Table 1. Fungal isolates used in this study.

Species	Strain identifiers	Mating type	Inoculum	Substrate	Location	References (if not this study)
Alternaria japonica	P400, ATCC 13618	—	—	Infected radish	Canada	Pryor and Gilbertson 2000; ATCC
Brachycladium papaveris	P351, CBS 116606*, Pf96	1/2	Conidium	*P. somniferum* (necrotic leaf)	Beltsville, Maryland	Farr et al. 2000; O'Neill et al. 2000
Brachycladium papaveris	P352, 19C	1/2	Conidium	*P. somniferum* (seeds from diseased pod)	Colombia	O'Neill et al. 2000
Brachycladium papaveris	P386, Colombia-1	1/2	Conidium	*P. somniferum* (seeds)	Huila, Colombia	Farr et al. 2000
Brachycladium papaveris	P387, 368263	1/2	Conidium	*P. somniferum* (seeds)	Turkey	
Brachycladium papaveris	P388, Ven	1/2	Conidium	*P. somniferum* (seeds)	Sierra de Perija, Venezuela	O'Neill et al. 2000
Brachycladium papaveris	P390, CBS 116609*, 414296	1/2	Conidium	*P. somniferum* (seeds)	Sweden	Farr et al. 2000
Brachycladium papaveris	P391, 414301	1/2	Conidium	*P. somniferum* (seeds)	Sweden	
Brachycladium papaveris	P411, Meffert No. 3, CBS 430.5	1/2	Conidium	*P. somniferum*	Germany	Meffert 1950
Crivellia papaveracea	P124, EGS 37-135, ZT9009	2	Ascospore	*Papaver* sp.	Schaffhausen, Switzerland	Crivelli 1983
Crivellia papaveracea	P128, CBS 116600*, EGS 37-134, ZT9008	1	Conidium	*Papaver* sp.	Schaffhausen, Switzerland	Crivelli 1983
Crivellia papaveracea	P349, 835-I-F2-1, ATTC 46989, CMI 246547	2	Conidium	*P. bracteatum* (stem)	Sunnyside, Washington	Farr et al. 2000; O'Neill et al. 2000
Crivellia papaveracea	P350, CBS 116605*, Cf96	2	Conidium	*P. somniferum* (necrotic leaf)	Beltsville, Maryland	Farr et al. 2000; O'Neill et al. 2000
Crivellia papaveracea	P354.1, CBS 116607*, 1841	1	Ascospore	*P. rhoeas* (stem)	Vienna, Austria	
Crivellia papaveracea	P354.8, CBS 116608*, 1841	1	Conidium	*P. rhoeas* (stem)	Vienna, Austria	
Crivellia papaveracea	P393, 415570	2	Conidium	*P. somniferum* (seeds)	India	
Crivellia papaveracea	P394, 414313	2	Conidium	*P. somniferum* (seeds)	Turkey	
Crivellia papaveracea	P395, 381488-1	2	Conidium	*P. somniferum* (seeds)	Iran	
Crivellia papaveracea	P396, CBS 116610*, 414263-1	2	Conidium	*P. somniferum* (seeds)	Hungary	
Crivellia papaveracea	P397, 414312	2	Conidium	*P. somniferum* (seeds)	Turkey	
Crivellia papaveracea	P398, 414263-2	2	Conidium	*P. somniferum* (seeds)	Hungary	
Crivellia papaveracea	P399, 414349	2	Conidium	*P. somniferum* (seeds)	Afghanistan	
Crivellia papaveracea	P412, Meffert No. 7359, CBS 208.50	2	Conidium	*P. somniferum*	Germany	Meffert 1950
Dendryphion nanum	P392, ATTC 16226	—	—	Oat field soil	Germany	Farr et al. 2000; ATTC
Ulocladium alternariae	P292, EGS 46-004, Pryor 31-41-05	—	—	Carrot (seeds)	USA	B.M. Pryor, personal communication

*Strains submitted to CBS in this study.

phaR, P351r (5′-GTC GGT GCC GAA CTC TCC G-3′), Jen2R, and PaM2r.

Phylogenetic analyses

The 18S, ITS and combined ITS, *EF* and *GPD* alignments were used for analyses. Phylogenetic analyses were performed with PAUP v.4.0b 10 (Swofford 2002) and MrBayes v3.0b4 for Bayesian analyses of phylogeny (Huelsenbeck and Ronquist 2001). Most parsimonious trees were inferred using 30 random addition replicates. Otherwise default settings were used and gaps were coded as missing data.

Maximum likelihood and Bayesian methods served to check parsimony results for a model or algorithm-specific bias. Most likely trees were inferred using default settings that included a transition:transversion rate set to "2", empirical base frequencies, invariable sites set to "0", and equal rates for all sites. For Bayesian analyses, a general time reversible model of evolution was used. Rate heterogeneity across sites was modeled with a gamma distribution. Four chains starting with a random tree were run for 1 million generations, retaining each 100th tree. The first 1000 trees of the 10 000 collected trees were discarded, and the subsequently calculated consensus trees were based on the remaining 9000 trees.

Bootstrap support for the branches was assessed using 500 bootstrap replicates, and default bootstrap settings except that taxa were added in a random sequence. To speed up parsimony bootstrap analyses of the single gene *Crivellia* data sets, a maximum of two representatives per allele were included. Preliminary parsimony analyses of a complete ITS data set had shown that bootstrap analyses aborted after 37 h and 102 replicates, gave almost identical bootstrap support values as analyses with a maximum of two identical DNA sequences included (data not shown). For combined parsimony analyses, as well as likelihood and Bayesian analyses, all taxa were included.

The partition homogeneity test (PHT) with 1000 replicates from the PAUP program was used to test data sets for conflicting phylogenetic signals. The permutation tail probability test as implemented in PAUP was used with 1000 replicates to test for the presence of a phylogenetic structure within a data set. For an explanation of these tests see Taylor et al. (1999).

The likelihood-based, nonparametric Shimodaira–Hasegawa test (SHT) (Goldman et al. 2000) was used as implemented in PAUP (Swofford 2002) with default settings except that the test was one-sided. The tree topology used for generation of constrained most parsimonious trees in PAUP was generated in MacClade v4.03 (Maddison and Maddison 2001).

Results

The results begin with the morphological taxonomic section to initially establish, in a formal manner, the names of the taxa as used throughout the text. The taxonomic concepts incorporate the evidence from phylogenetic analyses and investigations of mating systems, which are reported in a later section.

Taxonomy

***Crivellia* Shoemaker & Inderbitzin gen. nov.**

Ascomata solitaria vel caespitosa, superficialia, globosa vel depresso-globosa, glabra, castanea. Rostrum minutum, centrale, teretum, papilliforme, glabrum; ostiolum eperiphysatum. Paries e cellulae, pseudoparenchymatae; externae crassitunicatae, umbrinae; intus tenuitunicatae, luteolae. Physes numerosae, septatae. Asci numerosi, basales, cylindrici, bitunicati, brevi-pedicellati, 4- vel 8-spori. Ascosporae, uniseriatae vel biseriatae, teretae, ellipsoideae, acuminatae deinde hemisphaericae, rectae, luteae, eguttulatae, glabrae, vaginatae; transsepta constricta, tenuia, 3 in ordine 2:1:2; cellulae centrale, 0- to 1-vertiseptatae, curtae, crassae; filum germinale 1 vel 4, laterale. Heterothallica vel homothallica.

TYPUS NOMINIS GENERIS: *Cucurbitaria papaveracea* De Notaris (1863, p. 62, Tab. 60, Figs. 1–3).

ETYMOLOGY: We propose the new genus to commemorate the mycological contributions of Paolo Crivelli.

***Crivellia papaveracea* (De Not.) Shoemaker & Inderbitzin nov. comb.** Figs. 1–20, 27–30, 80

BASIONYM: *Cucurbitaria papaveracea* De Not. Sphaeriacei italici Centuria I. Fascicolo 2. R.I. De' Sordo-Muti. 1863. p. 62. Tab. 60. Figs. 1–3.

≡ *Pleospora papaveracea* (De Not.) Sacc. Syll. Fung. 2: 243 (1883) but not *Pleospora papaveracea* sensu Farr et al. (2000)

For additional synonyms see Shoemaker (1968).

DESCRIPTION: Ascocarps solitary to clustered on stems, superficial, globose to depressed-globose, collapsing in age, (250) 320–400 μm wide, 220–300 μm high, glabrous, dark brown (Fig. 27), interspersed with dark brown microsclerotia and macroconidiophores. Beak small, central, terete, papilliform, 20–30 μm long, 40–50 μm wide, glabrous, in section of 3–5 layers of polygonal 10–15 μm cells around a 20–25 μm ostiole without periphyses. Ascocarp wall in longitudinal section, laterally uniformly 25–50 (60) μm thick of 4–8 layers of isodiametric, 10–15 μm, pseudoparenchyma cells, outer cells thick-walled and dark brown, inner cells thin-walled, yellow. Physes numerous, 1.5–2 μm wide, with thin septa at 5–10 μm. Asci numerous, in a broad basal layer, from repeating croziers, with a "foot cell", cylindrical, bitunicate, (90) 100–120 × 7–12 (21) μm, short-stalked with 4, 6 or 8 uniseriate or biseriate ascospores (Figs. 29, 30). Ascospores terete, ellipsoidal, L/W 3.0, straight, 20–25 (27) × 6–9 μm, transversely 3-septate in sequence 2:1:2, with 1 vertical septum in either or both central cells, central cells shorter, first septum median (0.50), slightly constricted at septa, without dots at ends of septa, septa thin, second cell from apex enlarged towards middle, pale yellowish brown, without guttules, smooth, with 5–8 μm sheath (Figs. 1–3, 28). Ascospores initially ellipsoidal, often with small, acuminate, apical apiculus that appears darker coloured possibly from refraction, old ascospores more nearly hemispherical at apex.

COMMENTS: Ascospore germination laterally from any cell, but

usually only one (rarely two) cells involved, cells of ascospores much inflated with evident constrictions at septa.

Crivellia papaveracea resembles *Pleospora papaveracea* sensu Farr et al. (2000), but the two differ in their anamorphs.

The collection DAOM 230456 (P354) was chosen as epitype of *Cucurbitaria papaveracea*. The epitype was used as a substitute for the type in molecular investigations.

Crivellia papaveracea was formerly placed in the genus *Pleospora* in the Pleosporales (Shoemaker 1968), but differs morphologically from the type of *Pleospora*, *P. herbarum*. The sexual spores of *P. herbarum* are dilute medium brown in color, with seven transverse septa and one to two longitudinal septa in each cell (Simmons 1985). The sexual spores of *Crivellia* are yellow, three-septate with a septum in both central cells (Shoemaker 1968). The *Brachycladium*-type asexual state in *Crivellia* differs from the *Stemphylium*-type in *Pleospora* (Simmons 1985). The *Stemphylium* anamorphs have percurrent conidiophores producing dictyosporous, globoid conidia, with a concave base unlike the phragmosporous conidia of *C. papaveracea*.

HOST: *Papaver* spp. (Papaveraceae).

COLLECTIONS EXAMINED: *Cucurbitaria papaveracea* N. 39. in caulibus Papaveris exsicco. Garzola presso Como [Italy]. *Cesati*. XI [1]840 Det. Dntrs. HOLOTYPE ex RO (DAOM 115602); *Pleospora papaveracea* (De Not.) Sacc., on *Papaver rhoeas* stems, Austria, Vienna, collected by W. Jaklitsch 28 October 2001, (Jaklitsch 1841, P354), determined by W. J., P. Inderbitzin, EPITYPE UBC F14995, DAOM 230456. Single ascospore isolate "2" derived from epitype material DAOM 230456 and filed with DAOM 230456.

CULTURE COLLECTION ACCESSIONS: Submitted to CBS were the single ascospore isolates P354.1 (ex EPITYPE collection P354 (CBS 116607)) and P128 (CBS 116600).

OTHER ILLUSTRATIONS: Shoemaker (1968) as *Cucurbitaria papaveracea* TYPE (DAOM 115602): Ascospores (Fig. 2, page 1145, Figs. 55, 56 plate 2), conidia (Fig. 49, page 1146, Figs. 60, 61, plate 2), macroconidiophore stalk (Fig. 61, plate 2). Crivelli (1983), as *Pleospora papaveracea*, source material of strains ZT9008 and ZT9009 (P128, P124): Ascospores (Fig. A, page 51).

Brachycladium penicillatum Corda, Icon. Fung. 2: 14 (1838), Figs. 31–48, 81, 82

≡ *Dendryphion penicillatum* (Corda) Fr., Summa Veg. Scan. 2: 504 (1849)

For additional synonyms see Shoemaker (1968).

Macroconidiophores on the host arising from a globose cell 20–30 µm diam., or from a microsclerotium-like group of cells 40–60 × 20–35 µm (Figs. 5, 6, 38, 40, 41), erect, cylindric, straight, simple then branched 2–4 times near apex (Fig. 39), dark reddish brown becoming light yellowish brown towards tip, 400–600 × 10–20 µm, septate at intervals of 10–17 (25) µm, 4–6 µm diam near apex, producing terminal poroconidia, and forming a new apex by subterminal (sympodial) growth. Microconidiophores (in culture) arising from undifferentiated mycelium and from mycelial coils, straight or curved, cylindric, simple or branched towards apex, 30–60 × 4–6 µm, septate at intervals of 10–20 µm and forming a new apex by subterminal (sympodial) growth (Figs. 4, 42). Conidia cylindric, straight, hyaline to light reddish brown, unconstricted, smooth, 17–30 (35) × (4) 5–7 (8) µm, (avg. 29 × 6.6 µm) with 3–4 (6) transverse septa, (avg. 3.2) mean cell length 6.9 µm (Figs. 7, 31–37, 42–48, 80, 81), first-formed septum basal, septa appearing thick from plasmolysis of protoplasts in glycerine jelly mountant (Figs. 31–37) or thin when mounted in water (Figs. 7, 42–48, 80, 81), cells subequal, hilum 2–3 µm wide, included in contour with internal central perforation (atrium type of Alcorn (1983)), apical scar present on some conidia from secondary formation of conidia, germination lateral from any cell (Fig. 82). On the natural substrate, artificial medium and on sterilized stems of *Papaver* spp. numerous small sclerotia (microsclerotia) are produced (Figs. 6, 8–14, 15–20).

Microsclerotia of collection P354 on *Papaver rhoeas* rounded, elongate or of irregular shape, superficial, 20–100 × 25–75 µm, dark brown to black, pigmentation obscuring the septa. On corn meal agar (CMA) for the strains of *B. penicillatum* in Table 1 microsclerotium initials present at the edge of the colonies, formed in the aerial mycelium or immersed in the agar, consisting of hyaline cells, 1 to 9 in a row, secondary longitudinal, oblique or transverse septa formed in some cells, leading to rounded or irregular microsclerotia, 10–85 × 10–40 µm. Microsclerotium initials at first hyaline (Fig. 15), then dark substance secreted from the cell walls (Figs. 16, 17) forming warts at times, first translucent, then gradually obstructing view of the septa (Fig. 18). In 6-d-old colonies on CMA, microsclerotia at the center of the colonies light brown to black, translucent to opaque, verruculose to smooth. After 30 d on CMA, most microsclerotia dark brown to black and opaque, 30–95 × 20–65 µm resembling microsclerotia from the natural substrate (Figs. 6, 8–10). On vegetable juice (V8) agar microsclerotia similar in dimensions to CMA, light brown after six days, some warted (Fig. 19, 20), then darkening, after 30 d pigmented and opaque as on CMA. Microsclerotia and microsclerotium initials also observed on *Papaver somniferum* leaves inoculated with strain P350, microsclerotia 25–45 × 20–35 µm.

COMMENTS: Microsclerotia, macroconidiophores and conidia present on the holotype of *Cucurbitaria papaveracea* (DAOM 115602) (Fig. 6) match those of the holoype of *Brachycladium penicillatum* (DAOM 49356) (Fig. 8). The anamorph of collection DAOM 230456 (P354) was selected as epitype of *B. penicillatum,* included as representative in DNA investigations and filed as DAOM 230457.

Brachycladium penicillatum resembles *B. papaveris*. Morphological differences inferred from the 22 isolates studied are the microsclerotia present in *C. papaveracea* but absent in *B. papaveris*, and the macroconidiophore formation in *C. papaveracea* on the natural substrate. Microsclerotia are conspicuous, dark, pigmented groups of cells formed in the mycelium of *B. penicillatum* on the natural substrate, different agar media and on inoculated opium poppy leaves. Microsclerotia vary considerably in size and shape, are up to 100 µm long and 75 µm wide, rounded, elongate or of irregular outline (Figs. 6, 8–20). These findings agreed with Meffert (1950) who considered the presence of microsclerotia to be diagnostic for *B. penicillatum* strain P412, separating it from *B. papaveris*, for example strain P411 (Table 1).

On the natural substrate, *B. penicillatum* forms macroconidiophores that are rigid and erect, consisting of aggregated hyphae that are several 100 μm long (Fig. 5), with conidiogenous cell-bearing branches at the apex. *Brachycladium papaveris* does not form macroconidiophores. However, macroconidiophores are rarely formed in culture and it is unclear if they are always present in *C. papaveracea* on the natural substrate. According to Farr et al. (2000), addition of a sterile wheat culm to agar cultures led most consistently to the formation of macroconidiophores. If this method indeed worked constantly, it could be used together with microsclerotium formation to differentiate *B. penicillatum* from *B. papaveris*.

COLLECTIONS EXAMINED: *Brachycladium penicillatum* Corda, Ca. Mus. Gart. Dec. 6 (1837). in caul. Papaver TYPE ex PR (DAOM 49356); *Cucurbitaria papaveracea* N. 39. in caulibus Papaveris exsicco. Garzola presso Como [Italy]. *Cesati*. XI [1]840 Det. Dntrs. TYPE ex RO (DAOM 115602); *Pleospora papaveracea* (De Not.) Sacc., on *Papaver rhoeas* stems, Austria, Vienna, collected by W. Jaklitsch 28 October 2001, (Jaklitsch 1841, P354), determined by W. J., P. Inderbitzin, EPITYPE (UBC F14995, DAOM 230457); Single conidium isolate "2" derived from epitype material DAOM 230457 and filed with DAOM 230457; *Papaver somniferum* leaves inoculated with *B. penicillatum* strain Cf96 (P350) (UBC F14997); *B. penicillatum* strains P124, P128, P349, P393-P399, P412 (Table 1).

CULTURE COLLECTION ACCESSIONS: Submitted to CBS were the single conidium isolates P354.8 (ex EPITYPE collection P354, CBS 116608), P350 (CBS 116605) and P396 (CBS 116610).

OTHER ILLUSTRATIONS: Shoemaker (1968), as *Dendryphion penicillatum* TYPE (DAOM 49356): Conidia (Fig. 43, page 1146; Figs. 57, 58, plate 2 Shoemaker (1968) as *Pleospora papaveracea* TYPE (DAOM 115602): Conidia (Fig. 49, page 1146; Figs. 60–62, plate 2), macroconidiophore stalk (Fig. 61 plate 2). Farr et al. (2000) as *Dendryphion penicillatum* strain Cf96 (P350): Microconidiophore (Fig. 9, page 150), macroconidiophore (Figs. 10, 11, page 150), conidia (Fig. 6, page 148), microsclerotia (Fig. 7, page 148). Meffert (1950), as *"Dendryphium penicillatum* var. *sclerotiale* nom. nud." strain Meffert No. 7359 (P412): Macroconidiophores and conidia (Fig. 11, page 458), microsclerotia development (Fig. 12, page 459). Crivelli (1983), as *Dendryphion penicillatum* strains ZT9008 or ZT9009 (P128, P124): Macroconidiophore, conidia and microsclerotium (as chlamydospore) (Fig. B, page 51).

COMMENT: *Dendryphion* as currently constituted (Ellis 1971) is not monophyletic. We show below that *D. nanum* (Figs. 75, 78, 79) is not closely related to *B. penicillatum*. *Brachycladium penicillatum* (Figs. 4–7, 31–48, 80–82) is morphologically quite distinct from *D. comosum* Wallr., the type of *Dendryphion*, characterized by tree-like macroconidiophores (Figs. 76, 77) but distinguished by branched conidia (Figs. 70–74). Consequently, we reverted to *Brachycladium* as the appropriate genus for *B. penicillatum*; *B. penicillatum* is the type of the generic name *Brachycladium* Corda. *Brachycladium* is applied here to the anamorph of *Crivellia papaveracea* and to a second species, *B. papaveris.*

Four possibly confounding names require a brief mention. *Sphaeria brachycladii* Lacroix (Desmazières 1860, No. 786) is a nomen nudum (Shoemaker 1968). *Sphaeria papaveris* (Schumacher 1803) is a synonym of *Pleospora herbarum* fide Lind (1913, p. 229) (Shoemaker 1968). *Dendryphium penicillatum* (Corda) Fries var. *sclerotiale* in Meffert (1950) nom. nud. contravenes Art. 36 (no Latin diagnosis) (Anonymous 1951, p. 38). *Helminthosporium papaveris* P. Henn. (1905) nom. nud. lacks a published description and is not in contention as a prior homonym of *H. papaveris* Sawada.

***Brachycladium papaveris* (K. Sawada) Shoemaker & Inderbitzin comb. nov.** Figs. 21–26, 49–69

BASIONYM: *Helminthosporium papaveris* K. Sawada, Jour. Formosan Nat. Hist. Soc. Vol. VII, No 32: 129. T. 6 (1917); Translation: T. Tanaka, Mycologia 12(6): 329–330 (1920). Other publications: Bull. Agric. Exp. Stat., Govt. of Formosa No. 128 pp. 20–22, T. 7. (1918); Special Bull. Agric. Exp. Stat., Govt. of Formosa No. 19 pp. 653–655. T. 14. Figs. 9–10 (1919)

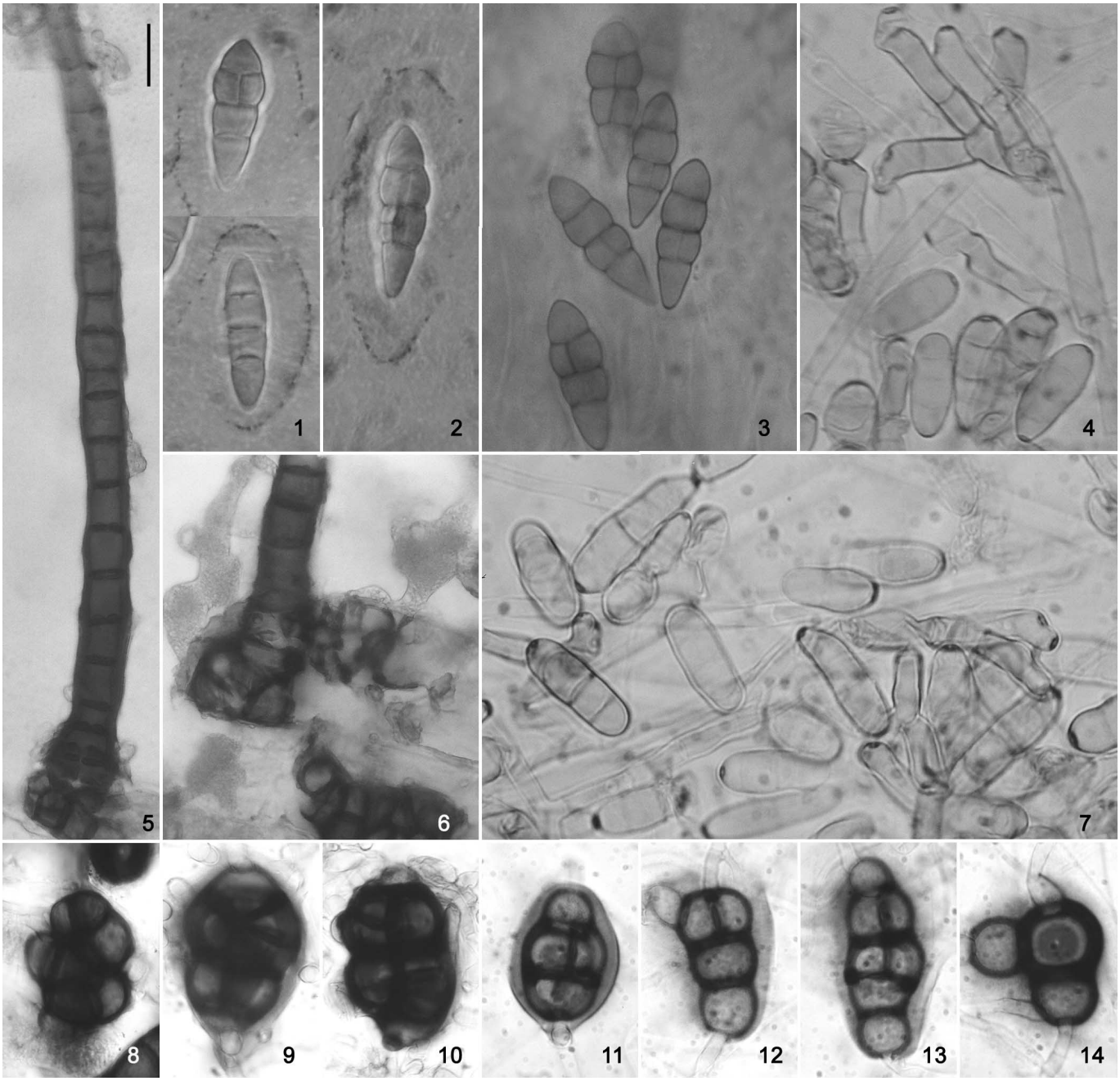
≡ *Dendryphion papaveris* (K. Sawada) K. Sawada, Descriptive Catalogue of Taiwan (Formosan) Fungi Part XI. College of Agric., Nat. Taiwan Univ., Taipei, Taiwan, China, Special Publication No. 8. p. 200 (1959)

DESCRIPTION: On brown spots on leaves, mainly hypophyllous. Mycelium internal, colourless, narrow, 2–4 μm diam., septate at 15–20 μm, microsclerotia absent, possible chlamydospores (Figs. 22, 24), ascoma initials (Figs. 25, 26) and cells excreting dark substance present (Figs. 21, 23). Conidiophores (micronematous) solitary or rarely 2–4 and divergent from adjacent bases, base of some a globoid cell, erect, slightly bent, branched once or twice, hyaline to pale brown, 20–130 × 4–5 μm (Figs. 55, 69). Conidiogenous cell terminal with one terminal, atrium-type scar or, by sympodial growth, several scars each at a geniculation, 15–20 × 4–5 μm (Fig. 69). Conidia blastogenous, solitary or rarely short-catenate from a terminal pore with or without intervening prolongation of distal cell, straight or slightly bent, nearly cylindrical, widest below middle, slightly tapered to base, more noticeably tapered to apex, rarely constricted at septa, light yellowish brown to medium reddish brown in age, ends broadly rounded, (25) 40–70 × 6–8.5 μm (avg. 53.6 × 7.7 μm), (3) 5–7 (8) septate (avg. 5.6), mean cell length 8 μm (Figs. 49–54, 56–68); scar atrium-type, 2–4 μm diam.; septa, thin, pigmented like the outer wall (euseptate), some (first-formed?) more prominent than others. Mode of germination not in evidence.

CULTURE STUDIES: Microsclerotia absent in culture. Somewhat similar structures present are mycelial cells excreting dark substance (Figs. 21, 23), thick-walled cells, possibly chlamydospores (Figs. 22, 24), and groups of thin-walled cells originating from lateral divisions in 30 d old cultures, possibly ascomata initials (Figs. 25, 26).

Cells excreting dark substance not always present, but formed by *B. papaveris* strain P351, present in culture after 6 d on CMA, immersed in agar, terminal or intercalary, one to seven cells in a row, 12–83 × 2.5–6.5 μm. Excreted substance deposited at the outside of the cell walls, outline of deposits not well defined, density decreasing from a central core towards the outside. Central cores dark brown to black, excreted uniformly and up to 2 μm wide, or deposited irregularly at several sites of a cell, up to 4 μm wide (Fig. 21), or

Figs. 1–7. Ascospores, microconidiophore, macroconidiophore, microsclerotium, and conidia. Scale bar = 10 µm. Figs. 1, 2, 5, 6. *Crivellia papaveracea*, TYPE, DAOM 115602. Figs. 3, 4, 7. *Crivellia papaveracea* P354, epitype, DAOM 230456. Figs. 1 and 2. Ascospores with sheath in water + India ink. Fig. 3. *Crivellia papaveracea* P354, epitype, DAOM 230456, ascospores in lactic acid. Fig. 4. *Brachycladium penicillatum* P354, epitype, DAOM 230457, single conidium isolate "2", PDA 22 °C 24 d, in lactic acid. Figs. 5 and 6. Macroconidiophores and microsclerotium in water. Fig. 7. *Brachycladium penicillatum,* epitype, DAOM 230457 (P354), single conidium isolate "2", PDA 22 °C 24 d, in lactic acid. **Figs. 8–14.** Microsclerotia. Scale bar = 5 µm. Fig. 8. *Brachycladium penicillatum*, TYPE, DAOM 49356, in glycerine jelly. Figs. 9 and 10. *Crivellia papaveracea,* epitype, DAOM 230456 (P354), single ascospore isolate "2", PDA 22 °C 24 d, in lactic acid. Figs. 11–14. *Brachycladium penicillatum*, epitype, DAOM 230457 (P354), single conidium isolate "2", PDA 22 °C 24 d, in lactic acid.

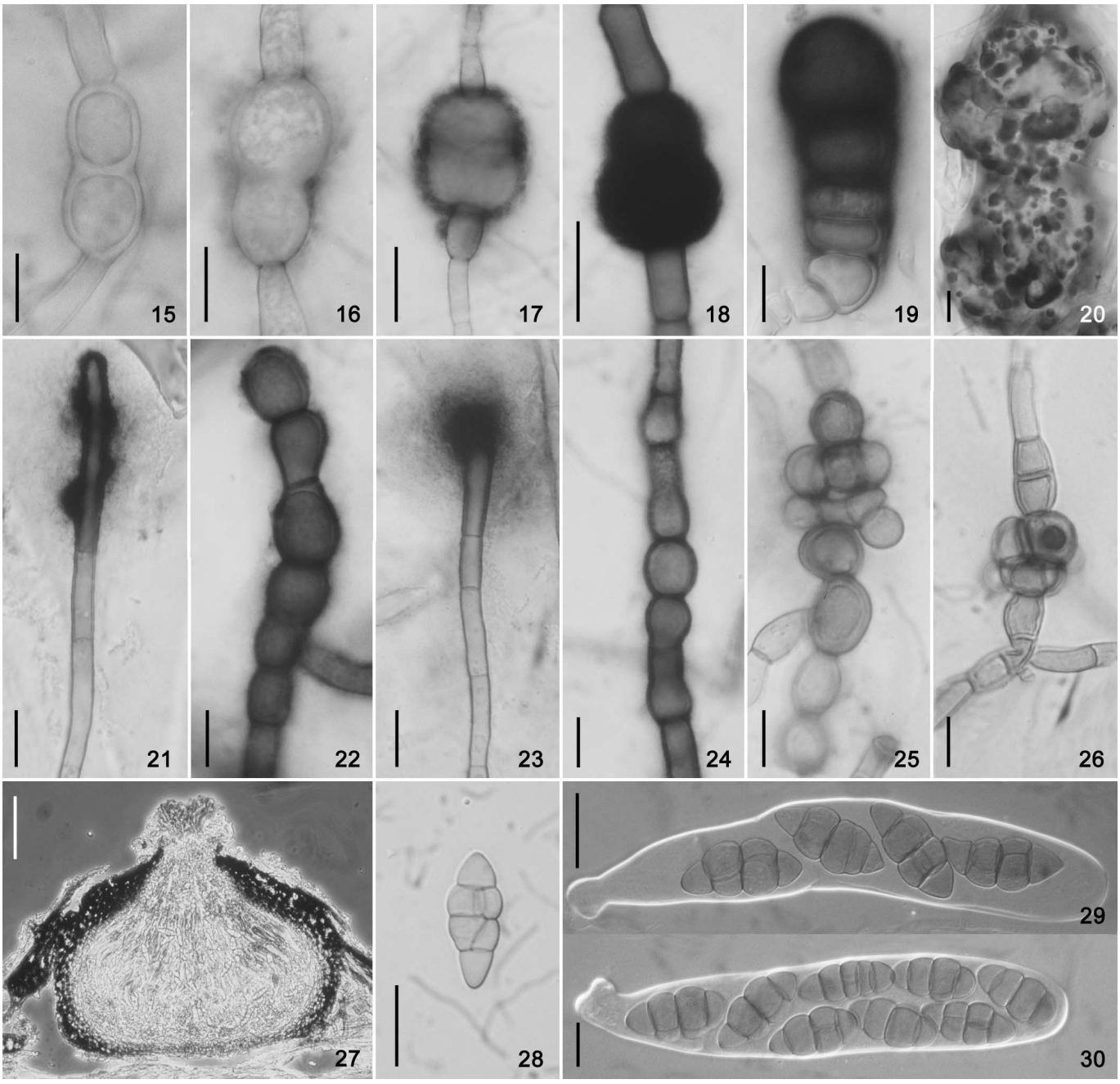


at the terminal end of a cell, forming a rounded structure up to 42 µm in diameter with a core of up to 10 µm in diameter (Fig. 23).

In isolate *B. papaveris* strain P351 after 6 d on CMA thick-walled, inflated or rounded mycelial cells present, possibly chlamydospores (Fig. 22, 24). Cells single or in chains, mostly intercalary, pigmented, brown, mostly remaining translucent, rounded to elongate, up to 15 µm wide.

Possible ascoma initials produced by *B. papaveris* strain P351 after 30 d on CMA, consisting of thin-walled, hyaline

Figs. 15–20. *Crivellia papaveracea* P354 microsclerotia (DAOM 230456). Scale bar = 10 μm. Figs. 15–18. 10 d old on CMA. Figs. 19 and 20. 6 d old on V8 agar. **Figs. 21–26.** *Brachycladium papaveris* P351 on CMA. Scale bar = 10 μm. Figs. 21 and 22. Melanized hyphae, 6 d old. Fig. 23. Melanized hypha tip 10 d old. Fig. 24. Melanized hypha 6 d old. Figs. 25 and 26. Ascoma initials, 30 d old. **Figs. 27–30.** *Crivellia papaveracea* P354 from *Papaver rhoeas*. Fig. 27. Ascoma wall, centrum and beak. Scale bar = 100 μm. Fig. 28. Ascospore. Scale bar = 10 μm. Figs. 29 and 30. Asci. Scale bar = 10 μm.



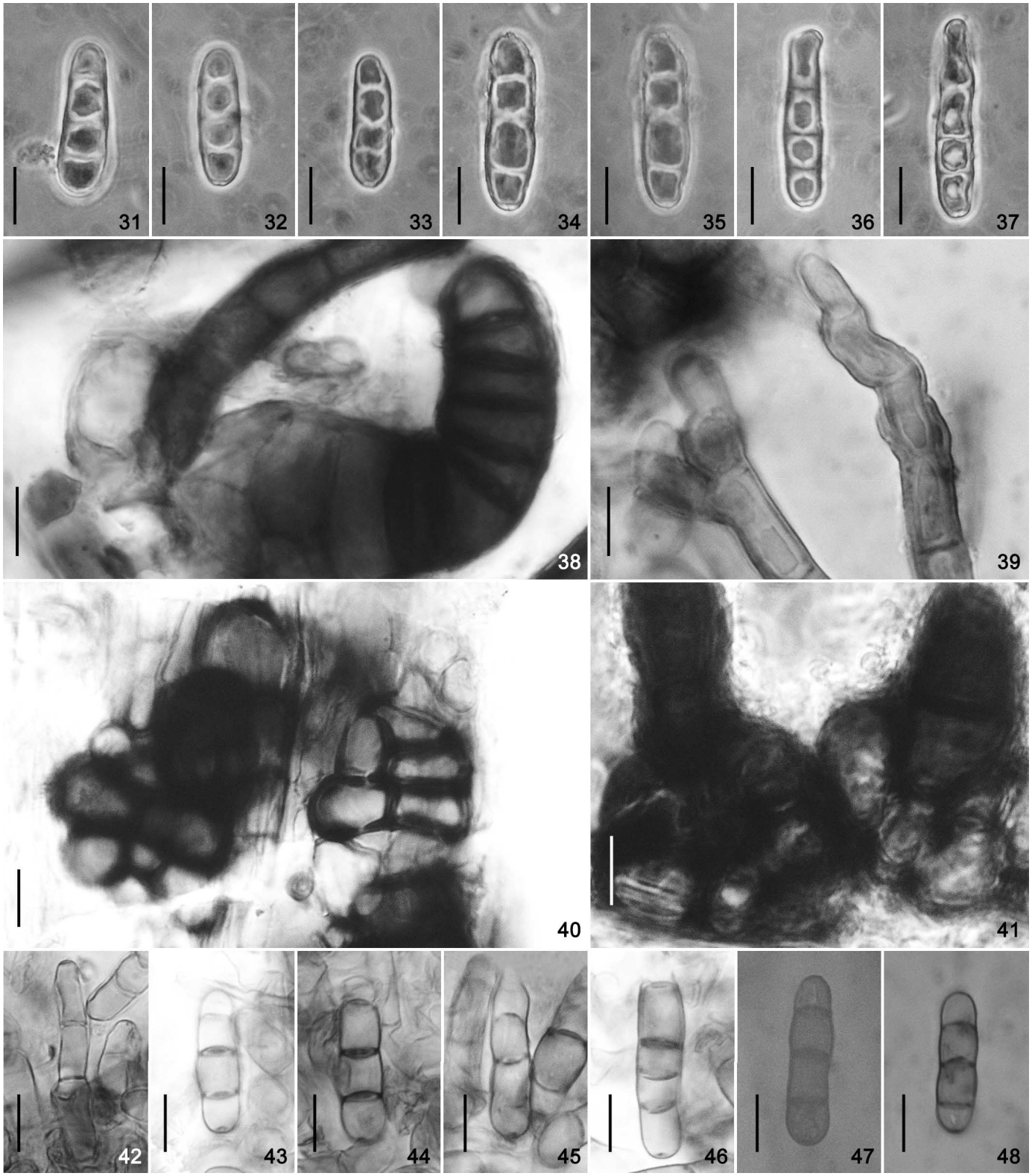
cells dividing in a plane parallel to the long axis of the hypha (Figs. 24, 25), up to ca. 20 μm wide when 2–3 cells in diameter.

COLLECTIONS EXAMINED: *Helminthosporium papaveris* Sawada, TYPE (BPI 443897); *Brachycladium papaveris* strain Pf96 (P351) EPITYPE (DAOM 233939), single conidium isolate from necrotic leaves of *Papaver somniferum*, collected and isolated by N. O. O'Neill, Beltsville, MD, USA (Table 1); *Papaver somniferum* leaves inoculated with *B. penicillatum* strain Pf96 (P351) (UBC F14996); *Brachycladium papaveris* strains P352, P386-P388, P390, P391, P411 (Table 1).

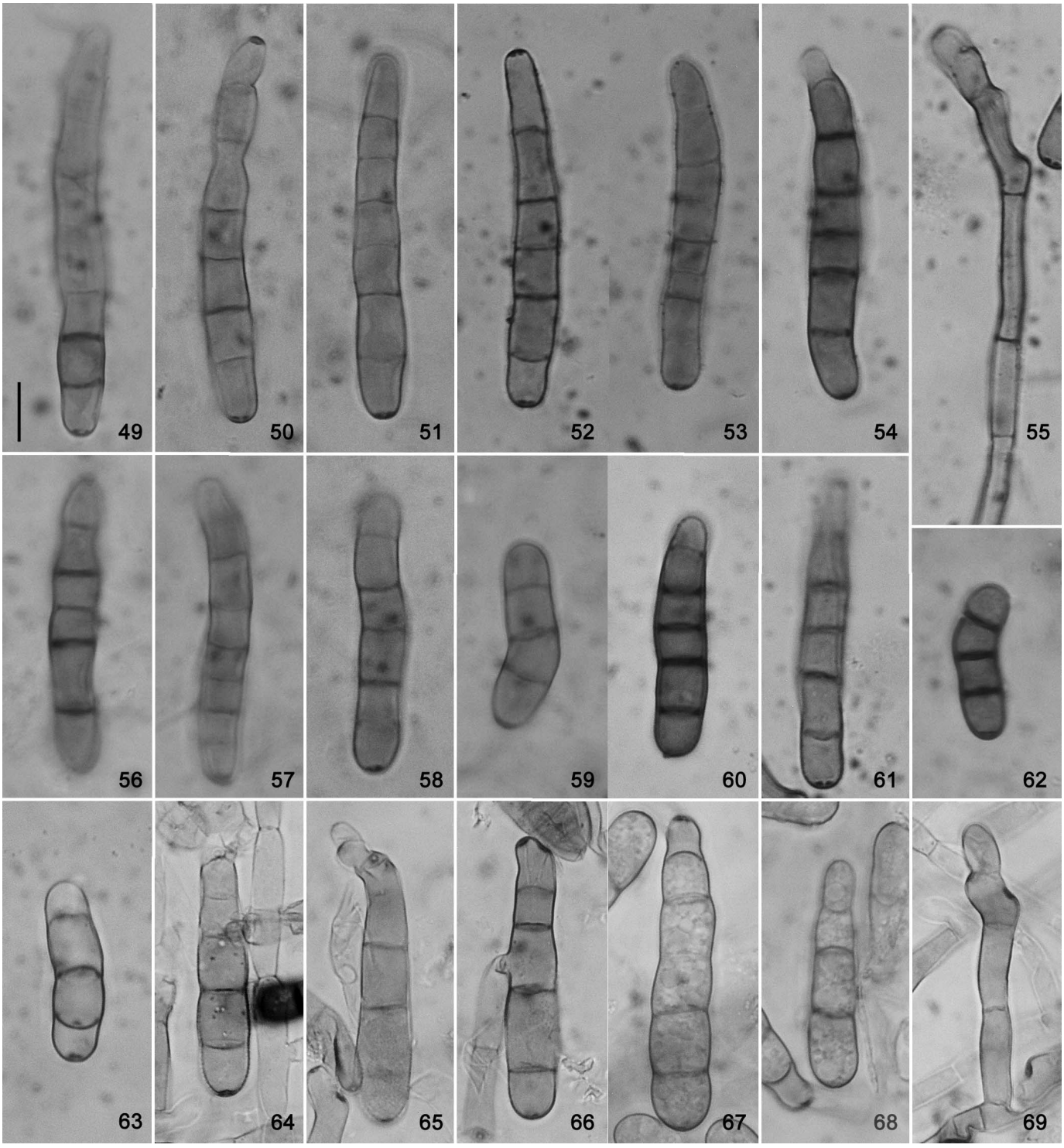
CULTURE COLLECTION ACCESSIONS: Submitted to CBS were single conidium isolates of *B. papaveris* strain P351 EPITYPE (CBS 116606) and strain P390 (CBS 116609).

COMMENTS: The recent culture of *Brachycladium papaveris* P351 (DAOM 233939) matches the type specimen of

Figs. 31–37. *Brachycladium penicillatum,* TYPE, DAOM 49356, 7 conidia (shrunken) and "chlamydospore" in glycerine jelly. Scale bar = 10 μm. **Figs. 38–41.** *Brachycladium penicillatum*, TYPE, DAOM 49356, conidiophore tips and bases in glycerine jelly. Scale bar = 10 μm. **Figs. 42–48.** *Brachycladium penicillatum*, from culture "*Dendryphium penicillatum* var. *sclerotiale*" nom. nud. CBS 430.50, P411, on V8 agar, conidiophore and 6 conidia, in water. Scale bar = 10 μm.



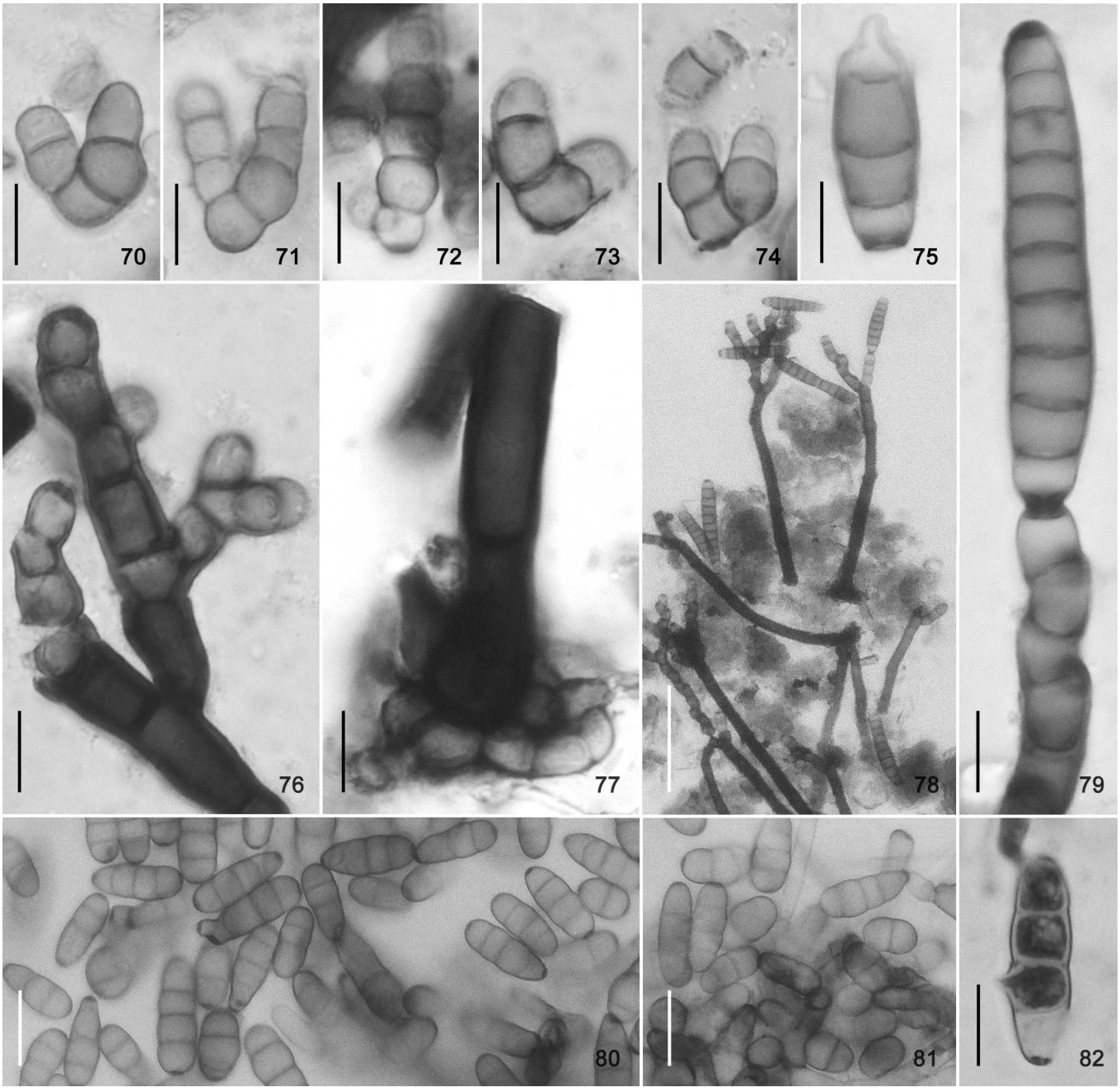
Figs. 49–62. *Brachycladium papaveris,* TYPE, BPI 44397 conidia and conidiophore in lactic acid. Scale bar = 10 μm. **Figs. 63–69.** *Brachycladium papaveris,* epitype, isolate Pf96, P351, DAOM 233939, six conidia and conidiophore from sterile alfalfa stem in water agar, in water. Scale bar = 10 μm.



H. papaveris Sawada and was chosen as an epitype for molecular studies.

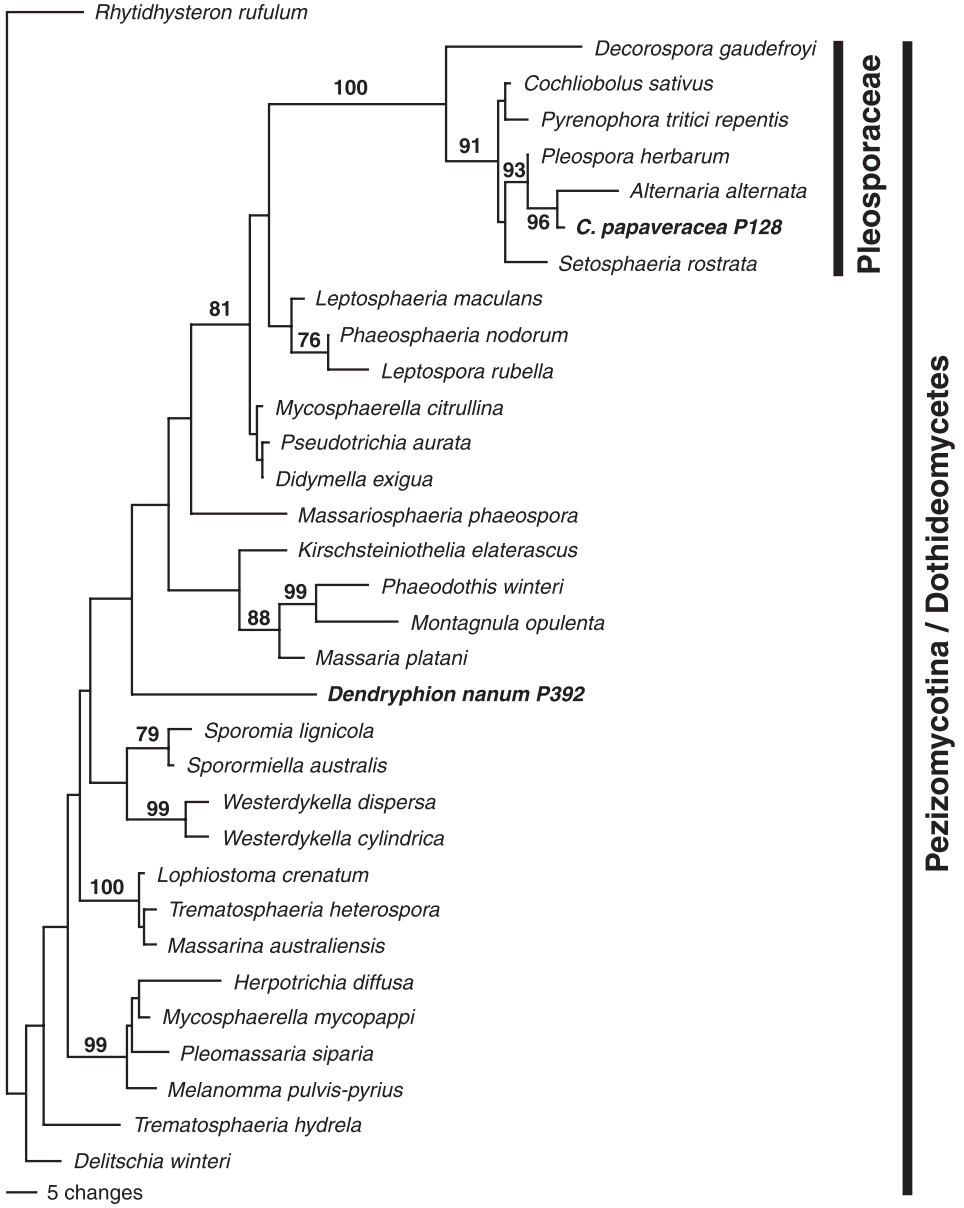
Brachycladium papaveris resembles *B. penicillatum*, but does not form microsclerotia. Instead, *B. papaveris* forms somewhat similar structures that might be confounded with microsclerotia at dissecting microscope view. These structures are chlamydospores, hyphal cells excreting a pigmented substance, and presumptive ascomata initials. Chlamydospores are rows of thick-walled cells present in

Figs. 70–74. *Dendryphion comosum*, TYPE, DAOM 41982, five branched conidia, in glycerine jelly. Scale bar = 10 μm. **Fig. 75.** *Dendryphion nanum* DAOM 233818, young conidium with truncate-concave scar, in lactic. Scale bar = 10 μm. **Figs. 76 and 77.** *Dendryphion comosum,* TYPE, conidiophore tips and base, in glycerine jelly. Scale bar = 10 μm. **Figs. 78 and 79.** *Dendryphion nanum* DAOM 233818, conidium and conidiophores, in lactic acid. Scale bar = 10 μm. **Fig. 80.** *Crivellia papaveracea,* epitype, DAOM 230456 single ascospore isolate "2", PDA, 22 °C, 24 d, in water. Scale bar = 10 μm. **Fig. 81.** *Brachycladium penicillatum,* epitype, DAOM 230457 single conidium isolate "2", PDA 22 °C 24 d, in water. Scale bar = 10 μm. **Fig. 82.** *Brachycladium penicillatum,* epitype, DAOM 230457 single conidium from host germinated on PDA 20 °C 20 h, cotton blue in lactic acid. Scale bar = 10 μm.



the mycelium of *B. papaveris* (Farr et al. 2000; Meffert 1950). However, as opposed to microsclerotia, chlamydospores are single rows of thick walled cells remaining translucent and are less than 20 μm wide (Figs. 22, 24). Another set of structures observed in the mycelia of *B. papaveris* were hyphal cells excreting a pigmented substance, as microsclerotia do in *C. papaveracea* (Figs. 21, 23). However, unlike microsclerotia, the excreting cells remain below 10 μm in diameter. Finally, 30 d old cultures of *B. papaveris* contain groups of thin-walled cells that appear to have originated from cell divisions perpendicular to the hyphal main axis (Figs. 25, 26). These structures are similar to ascomata initials illustrated in a developmental study of a homothallic opium poppy fungus (Schmiedeknecht 1962).

Fig. 83. Placing *Crivellia papaveracea* isolate P128 and *Dendryphion nanum* P392 within the ascomycetes using 18S rDNA analyses. Shown is one of 16 most parsimonious trees (393 steps, CI = 0.667, RI = 0.800) obtained from phylogenetic analyses based on TreeBASE alignment M1158 supplemented with sequences of *C. papaveracea* isolate P128, *D. nanum* P392, and the three closest BLAST matches for *D. nanum*, viz. *Trematosphaeria heterospora* (AY016354), *Westerdykella cylindrica* (AY016355), and *Mycosphaerella mycopappi* (U43463) from GenBank. For details of remaining taxa including GenBank Accession numbers, see Inderbitzin et al. (2002). Sequences obtained in this study are in bold. Bootstrap support percentages above 70 are by the branches. *Crivellia papaveracea* isolate P128, a representative of *Crivellia*, clusters with 96% bootstrap support with *Alternaria alternata*, within the Pleosporaceae. The large-spored *D. nanum* P392 is not closely related to *C. papaveracea*, and is not close to any of the taxa included in the analysis.

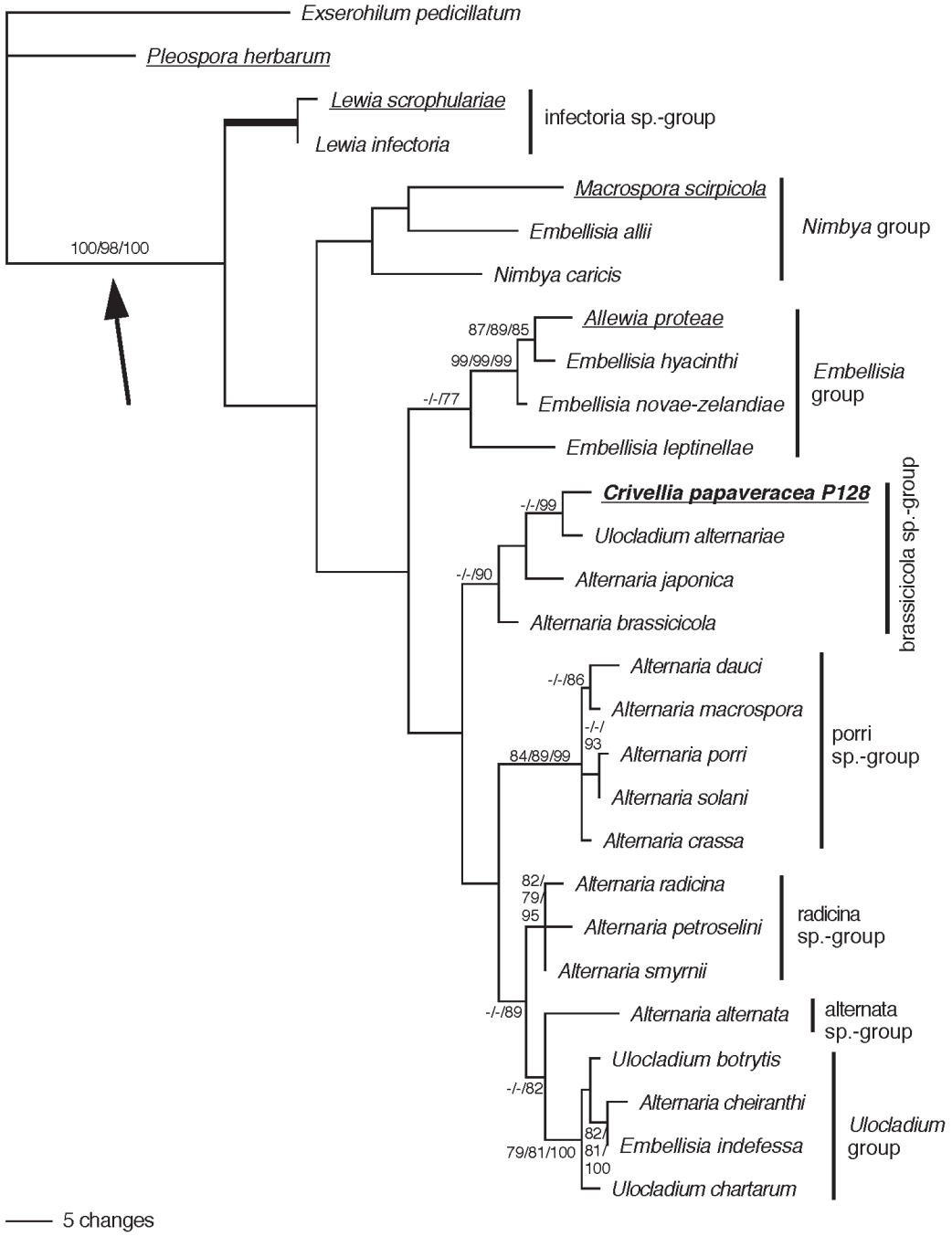


The presumptive ascomata initials in *B. papaveris* can be distinguished from microsclerotia by their thin, translucent walls and regular cell arrangement.

OTHER ILLUSTRATIONS: Sawada (1919), as *Helminthosporium papaveris* BPI 443897 TYPE: Conidiophore (Fig. 9), conidia (Fig. 10). Farr et al. (2000), as *Dendryphiella* sp. strain Pf96 (P351), epitype: Conidia (Fig. 4, p. 148), conidiophore (Fig. 8, p. 150), chlamydospores (Fig. 5, p. 148). Meffert (1950), as *Helminthosporium papaveris*: chlamydospores (Fig. 2, page 446), conidiophores (Fig. 3, pages 447), chains of conidia (Fig. 4, page 448), conidia (Fig. 5, page 449; Fig. 7, page 451).

OTHER DESCRIPTIONS: Tanaka (1920) published a translation of the original description by Sawada (1917) as follows: Conidiophores fasciculate or solitary, copiously branched, cylindric, many septate, yellowish brown, 86–130 × 6–7 μm, terminating with a single conidium, after its abstriction a second conidium is formed. Conidia cylindric, both ends

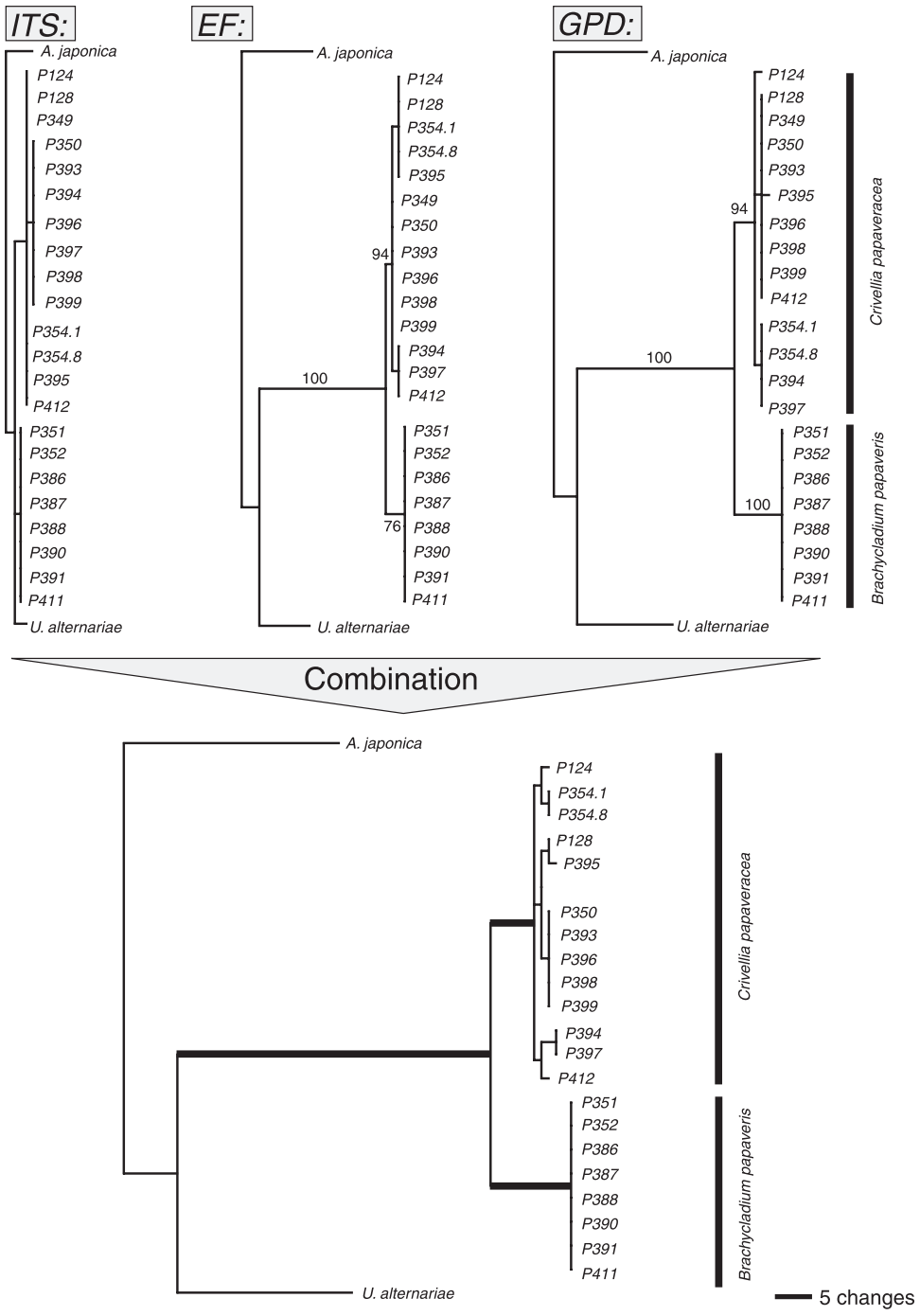
Fig. 84. Placing *Crivellia papaveracea* isolate P128 within *Alternaria* and related genera. ITS data confirmed results from 18S rDNA analyses. Shown is one of two most parsimonious trees obtained from phylogenetic analyses based on a modified ITS rDNA TreeBASE alignment M1585 by Pryor and Bigelow (2003) to which *C. papaveracea* isolate P128 and *Lewia scrophulariae* were added. The sequence obtained in this study is in bold, representatives of teleomorph taxa are underlined. Bootstrap support percentages above 70 are by the branches for parsimony, likelihood and Bayesian analyses in this order. Branches in bold were supported by 100% in all analyses. Group designations follow Pryor and Bigelow (2003). The branch supporting the *Alternaria* group is marked by an arrow. Note that *C. papaveracea* isolate P128 is nested within the *Alternaria* group, distinct from *Pleospora herbarum*. A Shimodaira–Hasegawa test confirmed that constraining *Pleospora* to be monophyletic yields a significantly worse tree (see text for details).



blunt, 3–10 septate, constricted, yellowish brown, 22–112 × 7–11 µm.

Examination of Sawada's (1919) illustrations (Figs. 9, 10) yielded the following features: conidiophores solitary or grouped, micronematous (not much wider than hyphae), yellowish-brown, branched from just below septa, septa thin, at intervals of 8–30(40) µm, conidiogenous cells terminal, producing one conidium through a flush, terminal pore, cicatrized, scar 2–4 µm diam., proliferation sympodial leaving a geniculate apical region, yellowish-brown, cylindric, 50–112 × 6–7 µm. Conidia solitary, blastic, cylindrical, broadly rounded at ends, straight, yellowish-brown, (2) 3–5 (10)

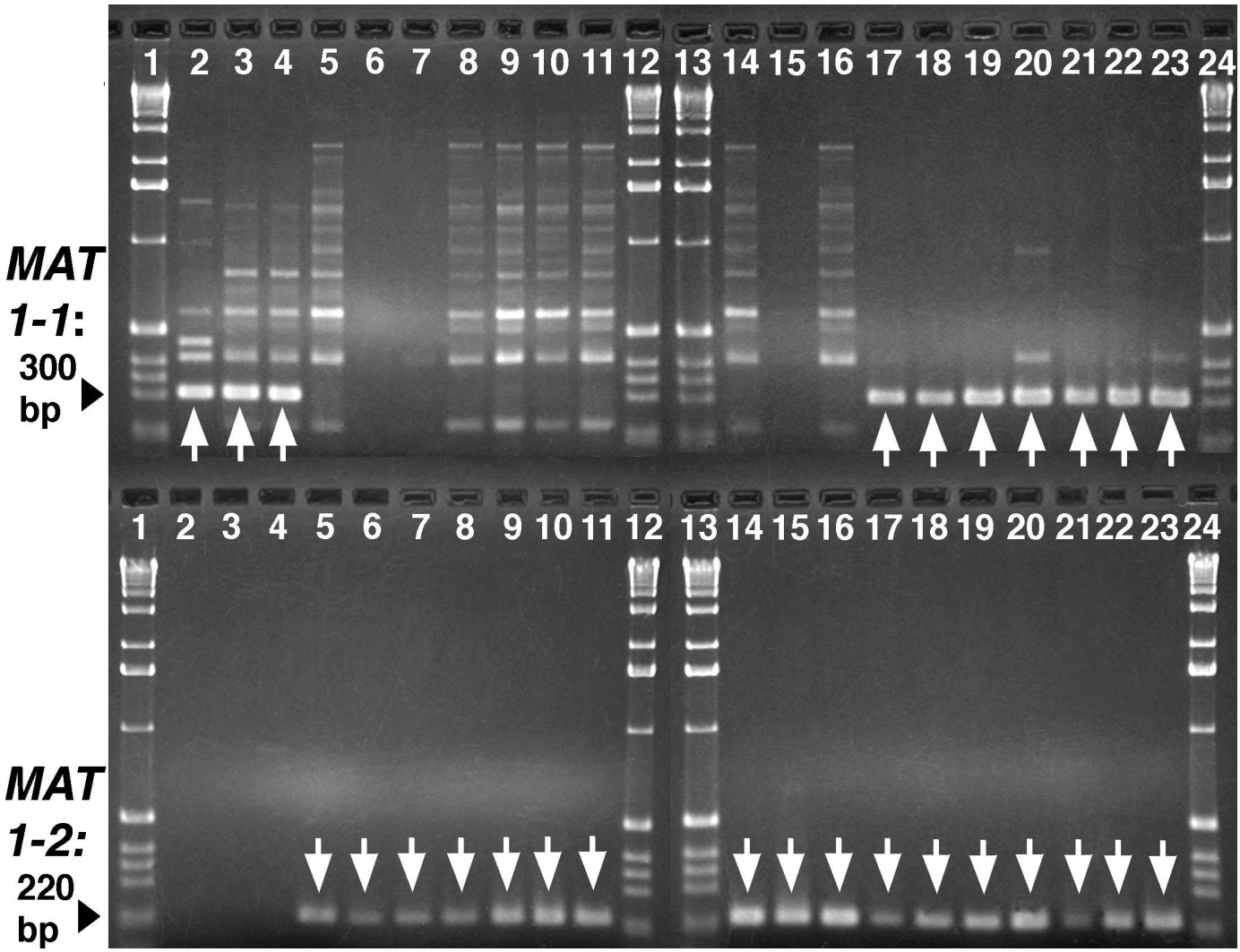
Fig. 85. Single and combined gene phylogenies of *Crivellia* and outgroups. Horizontal branch lengths are to scale and proportional to changes along the branches, to illustrate differing information content between the datasets. Phylogenies based on ITS, *EF*, and *GPD* data sets are shown in top half, a phylogeny based on a combined analysis of all three data sets is shown at the bottom. All phylogenies are most parsimonious trees. For the single gene trees, parsimony bootstrap support percentages above 70 are shown by the branches, except for ITS where *Brachycladium papaveris* is supported by 65, and *Crivellia papaveracea* by 91% of the bootstrap replicates. In the tree from combined analyses, branches in bold were supported by 100% of the replicates in parsimony, likelihood, and Bayesian analyses, except for *C. papaveracea* supported by 99% of the parsimony replicates. Note that all trees recognize both *C. papaveracea* and *B. papaveris* to be monophyletic, and all but the ITS tree supports a common origin of *C. papaveracea* and *B. papaveris* with 100% bootstrap support.



septate at intervals of (7) 8–9 (15) µm, avg. 10 µm, constricted slightly at septa, with a flush, basal scar 2–4 µm diam.

There was good agreement between the original description, later illustration, and the observations noted from a re-examination of BPI 443897. The conidiophores were micronematous about the same width as the subtending mycelium, often solitary, divergent from a common base when aggre-

Fig. 86. Screening of *Crivellia* isolates for presence of mating type genes *MAT1-1* and *MAT1-2*. Agarose gels showing results of *MAT1-1* and *MAT1-2* screening using specific primer pairs PaAlphaF/PaAlphaR and Jen2F/Jen2R (Fig. 87). All 22 *C. papaveracea* and *B. papaveris* isolates used for ITS, *EF,* and *GPD* phylogenetic analyses were screened with both primer sets. Top gels show results from *MAT1-1* screening, bottom gels from *MAT1-2* screening. Lanes 1, 12, 13, and 24 are DNA ladders; the remaining lanes are isolates P124 to P399 in ascending order (see Table 1). White arrows indicate bands of expected size of ca. 300 bp for *MAT1-1*, and ca. 220 bp for *MAT1-2*. Lanes 2–4 show isolates with *MAT1-1* only, lanes 5–16 show isolates with *MAT1-2* only, and lanes 17–23 show isolates with both *MAT1-1* and *MAT1-2* specific bands. To confirm the identity of the PCR bands, the following isolates were PCR amplified for DNA sequencing using a forward primer on the common ORF1, and a mating type specific reverse primer: *Crivellia papaveracea* isolate P128 (lane 3), *B. papaveris* isolate P390 (lane 16), and *C. papaveracea* isolate P396 (lane 22). The following two isolates were not included on the gels: *Crivellia papaveracea* isolate P412 had a typical *MAT1-2 band*, and *B. papaveris* isolate P411 had typical *MAT1-1* and *MAT1-2* bands.



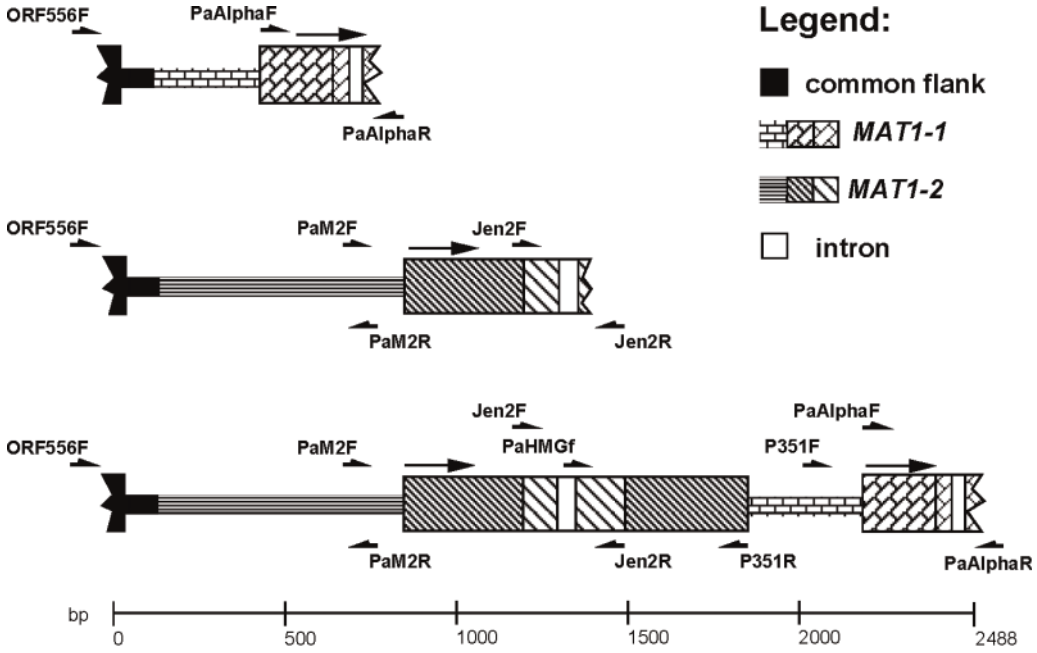
gated. The conidiogenous cells developed sympodially by subterminal growth below the apical conidium. The conidium scar is the atrium-type as defined by Alcorn (1983). The conidia are euseptate with a thin but clearly defined wall like the wall of the conidium. The septa are quite distinct in older, darker conidia but constrictions at the septa were rarely noted in contrast to Tanaka's observation that the conidia were constricted. The dimensions were in good agreement with conidia mostly 7–8 μm wide and the mean cell length 8 μm.

TELEOMORPH: Meffert (1950) and Farr et al. (2000) illustrated a *Crivellia*-type teleomorph possibly of *B. papaveris*. Owing to lack of voucher material and failure to induce teleomorph formation, we were unable to formally describe a teleomorph for *B. papaveris*. For illustrations see Meffert (1950): Ascoma, asci and ascospores (Fig. 9, page 454); ascomata surface view (Fig. 10 bottom, page 455) (as *Pleospora papaveracea*); Farr et al. (2000): Ascoma section (Fig. 1, page 148), asci (Fig. 2, page 148), ascospores (Fig. 3, page 148) (as *Pleospora papaveracea* strain Pf96, P351).

The phylogenetic position of Crivellia *within the ascomycetes*

The 18S ribosomal DNA (18S rDNA) sequence of *C. papaveracea* isolate P128 was compared with all other

Fig. 87. Diagrams of sequenced mating type regions. Short arrows indicate positions of primers used. From top to bottom: ORF1 – *MAT1-1* (from *Crivellia papaveracea* isolate P128), ORF1 – *MAT1-2* (from *C. papaveracea* isolate P396), and ORF1 – *MAT1-2* – *MAT1-1* (from *Brachycladium papaveris* isolate P390). Scale at bottom in basepairs refers to all diagrams. Wide boxes indicate genes, with directions of transcription marked by long arrows above genes, narrow boxes are intergenic regions or portions of the idiomorphs. Shark teeth indicate incompletely sequenced ORF. Empty boxes are introns. In all diagrams, filled black boxes are the 5′ common flanking regions, consisting of ORF1 and part of intergenic region. The idiomorphs are filled with either bricks for *MAT1-1* or lines for *MAT1-2*. For *MAT1-1*, slanted bricks are the *MAT1-1* gene containing the DNA binding alpha box motif (light-colored bricks). For *MAT1-2*, slanted lines are the *MAT1-2* gene containing the DNA binding HMG motif (spaced lines). The *MAT1-2* gene in *B. papaveris* isolate P390 is missing 43 bp at the 3′ end, and is fused to the 5′ end of a *MAT1-1* idiomorph.



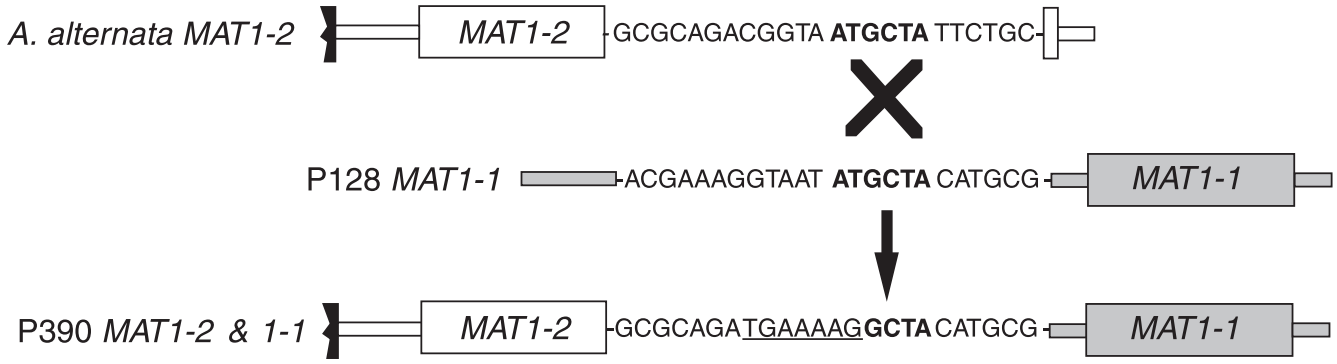
sequences in GenBank using a BLAST search. The closest matches with equal score and E-value were 18S rDNA sequences of *Alternaria raphani* J. W. Groves & Skolko, *A. brassicicola* (Schw.) Wiltshire, and two strains of *Pleospora herbarum* supporting morphological data that *C. papaveracea* is likely a member of the Pleosporales. To determine a closest relative of *C. papaveracea*, the 18S rDNA alignment M1158 from Inderbitzin et al. (2002) containing taxa of *Alternaria* and *Pleospora* was retrieved from TreeBASE. The TreeBASE alignment comprised 1720 characters and 28 taxa, including *Pleospora herbarum* and *Alternaria alternata* (Fr.:Fr.) Keissler, as well as a representative selection of the Pleosporales with *Rhytidhysteron rufulum* (Spreng.) Speg. as outgroup. The *C. papaveracea* isolate P128 rDNA sequence was 1629 bp in length and was added to the TreeBASE alignment resulting in an alignment of 29 taxa and 1720 characters. Parsimony analyses yielded one most parsimonious tree (MPT), measuring 350 steps. Apart from the addition of *C. papaveracea* isolate P128, the MPT was identical in topology to the tree obtained using alignment M1158 in Inderbitzin et al. (2002). *Crivellia papaveracea* isolate P128 grouped with *A. alternata*, supported by a high bootstrap support percentage of 96 (data not shown, but see Fig. 83).

The phylogenetic position of the presumptive Crivellia *relative* Dendryphion nanum

Dendryphion nanum is morphologically similar to the asexual state of *C. papaveracea* and *B. papaveris*, and has been considered a close relative to *Crivellia* (Farr et al. 2000). *Dendryphion nanum* has a similar conidiophore (Fig. 78), but the conidia are darker, more highly septate, 10–12 µm wide, and up to 90 µm long (Figs. 75, 79), and considerably longer than either *B. papaveris* or *C. papaveracea* conidia that have approximate mean lengths of 45 and 15 µm, respectively (Farr et al. 2000). The closest BLAST search matches to the *D. nanum* 18S rDNA sequence were *Trematosphaeria heterospora* (AY016354), *Westerdykella cylindrica* (AY016355), *Mycosphaerella mycopappi* (U43463), and *Sporormia lignicola* Phillips & Plowr. of the Pleosporales. The first three of these sequences, along with the 1634 bp 18S rDNA sequence of *D. nanum*, as well as the *C. papaveracea* isolate P128 18S rDNA sequence were added to the 18S TreeBASE alignment M1158. The resulting alignment comprised 33 taxa, and 1721 characters. Phylogenetic analyses yielded 16 most parsimonious trees (MPTs) of 393 steps.

Based on phylogenetic analyses, *D. nanum* is a member of the Pleosporales with uncertain affinity (Fig. 83). *Dendryphion nanum*'s phylogenetic position is basal to the group from *Massaria platani* Cesati to *Decorospora gaudefroyi* (Pat.) Inderb., Kohlm. & Volkm.-Kohlm., below 60% bootstrap support (Fig. 84). To find a closest relative would require extensive morphological and phylogenetic analyses that were beyond the scope of this paper. Partial 18S, ITS, *EF*, and *GPD* DNA sequences of *D. nanum* were submitted

Fig. 88. Diagram illustrating the hypothetical crossover event leading to the fused *MAT1-2 – MAT1-1* regions at *MAT* in homothallic *Brachycladium papaveris* isolate P390 (AY382474). *Alternaria alternata MAT1-2* (AB005039), used here as a *Crivellia papaveracea MAT1-2* surrogate (white), and *Crivellia papaveracea MAT1-1* from isolate P128 (AY377898) represent possible heterothallic ancestral *MAT* regions. DNA sequences shown correspond to positions 2094-2117, 66-89, and 1872-1895 in the respective GenBank accessions. *Alternaria alternata MAT1-2* shares the presumptive crossover site motif, ATGCTA (bold), with *C. papaveracea MAT1-1*. In the fused *MAT* region of *B. papaveris*, four nucleotides of the motif are present; however, seven nucleotides (underlined) are not found in the presumptive ancestral sequences.



to GenBank (AY382475, AY387657, AY383601, AY386329). No analyses are presented here involving the data from *D. nanum* protein coding genes.

The phylogenetic position of Crivellia *among* Alternaria *and related genera*

To further investigate the generic affinity of *C. papaveracea* isolate P128, the ITS alignment M1585 containing *Embellisia*, *Nimbya*, *Alternaria*, *Ulocladium*, and *Stemphylium* species was retrieved from TreeBASE. The alignment was 632 characters long and contained 40 taxa. We reduced the taxon sampling to one representative per ITS allele, except for the *Stemphylium* and infectoria species-groups (Pryor and Bigelow 2003), where only one representative of each group was retained. Added to the alignment were the type of *Lewia*, *L. scrophulariae* (Simmons 1986) from GenBank, as well as the *C. papaveracea* isolate P128 ITS sequence (556 bp in length) resulting in a 28 taxon, 631 character dataset. Parsimony analysis yielded two most parsimonious trees of 262 steps, differing in the placement of *Ulocladium botrytis* Preuss, which was either sister taxon to the lineage containing *Alternaria cheiranthi* (Libert) Bolle, or placed in a polytomy with the latter and *U. chartarum* (Preuss) Simmons (data not shown). The most parsimonious tree topologies obtained here were congruent on a 70% bootstrap support level with Fig. 84 in Pryor and Bigelow (2003) for taxa included in both studies. The added taxa *L. scrophulariae* and *C. papaveracea*, grouped with *L. infectoria* and *Ulocladium alternariae*, respectively (Fig. 84).

Phylogenetic testing

Crivellia *is not a member of* Pleospora

In parsimony analyses, *Crivellia papaveracea* strain P128 and *Pleospora herbarum* from the modified ITS alignment in Pryor and Bigelow (2003) were constrained to be monophyletic resulting in 45 most parsimonious trees of 282 steps each. The constrained trees were thus 20 steps longer and all significantly worse ($P < 0.05$ based on the Shimodaira–Hasegawa test; Goldman et al. 2000) than the unconstrained most parsimonious trees.

Phylogenetic species of Crivellia

The single-locus datasets ITS, *EF*, and *GPD* were analyzed with parsimony. DNA sequences used were submitted to GenBank as AY376639–AY376662, AY375367–AY375390, and AY376326–AY376349. Based on ITS results (Fig. 84), we chose *A. japonica* and *U. alternariae* as outgroups. Topologies showing bootstrap support for one most parsimonious tree from each dataset are shown in Fig. 85. The ITS alignment was 474 characters in length; 14 characters were variable (3.0%), and 6 were parsimony informative (1.3%). The two most parsimonious ITS trees were 15 steps in length (data not shown, but see Fig. 85). The *EF* data set of 24 taxa comprised 831 characters, of which 43 were variable (5.2%) and 22 were parsimony informative (2.6%). The *EF* data set resulted in one most parsimonious tree, 48 steps long. The *GPD* data set comprised 24 taxa and 471 characters, of which 66 of the characters were variable (14.0%) and 35 characters were parsimony informative (7.4%). The one most parsimonious *GPD* tree was 72 steps long (Fig. 85).

Visual inspection showed that the most parsimonious trees based on ITS, *EF*, and *GPD* data sets were substantially congruent (Fig. 85). All trees supported the monophyly of *C. papaveracea* and *B. papaveris*. *EF* and *GPD* trees both backed the monophyly of the *C. papaveracea* and *B. papaveris* isolates with 100% support, whereas ITS could not resolve the branching order. As a statistical test of congruence, we used PHT with the null hypothesis of no difference among the data sets. Data sets were considered significantly incongruent if PHT was $P < 0.001$ (Cunningham 1997). Four homoplasious characters contained in the single data sets, as well as 1713 non-informative characters were removed for PHT (Cunningham 1997; Kroken and Taylor 2001). Thus the single data sets contained 59 characters (4, 22, and 33 for ITS, *EF*, and *GPD*

data sets, respectively). To expedite the analysis, six of the eight *B. papaveris* isolates with identical genotypes were excluded, leaving 18 taxa in the analyses. The PHT could not reject the null hypothesis of congruence between the data sets ($P = 0.015$). A similar P value was obtained with the 1713 uninformative characters included ($P = 0.048$). When the four homoplasious characters were added, the P value was much lower ($P = 0.004$), or $P = 0.002$ when the 1642 uninformative characters were excluded as well. However, this was still above the level of significance of $P < 0.001$ recommended by Cunningham (1997). This sensitivity of the PHT to homoplasy was also observed by Barker and Lutzoni (2000). Thus, the three data sets were judged to be suitable for combined phylogenetic analyses.

The combined ITS, *EF*, and *GPD* data set contained 1776 characters, 123 characters were variable (6.9%), of which 63 were parsimony informative (3.5%). The resulting 22 most parsimonious trees were 140 steps long (data not shown). The monophyly of *C. papaveracea* plus *B. papaveris* was supported by 100%, *C. papaveracea* by 99%, and *B. papaveris* by 100% (Fig. 85). Within *C. papaveracea*, only isolates P394 and P397 received bootstrap support above 70%. The most parsimonious trees differed in topology within *C. papaveracea*: Only isolates P394 and P397 grouped together in all most parsimonious trees (data not shown).

Maximum likelihood and Bayesian analyses also supported monophyly of *C. papaveracea* plus *B. papaveris*, *C. papaveracea* and *B. papaveris* (Fig. 85).

Testing for a history of recombination in Crivellia papaveracea

Although PHT failed to detect significant conflict between the ITS, *EF*, and *GPD* datasets, some evidence for conflict was present. When ITS, *EF*, and *GPD* datasets were combined in one parsimony analysis, the most parsimonious trees were 132 steps in length, four steps longer than the sum of the trees inferred from single data sets. The four additional steps were caused by homoplastic changes within *C. papaveracea* (data not shown).

To test for panmixia in *C. papaveracea*, the permutation tail probability test (Burt et al. 1996) was used. We restricted our data set to isolates within *C. papaveracea* with unique DNA sequences to expedite analyses, and excluded all uninformative characters resulting in a dataset of eight taxa and five characters. The test result indicated that the null hypothesis of panmixia could not be rejected ($P = 0.87$). Because of the limited sample size and low number of characters used, definite proof of the presence of a history of recombination within *C. papaveracea* was not possible. However, we have proof for extant meiotic segregation of mating type alleles in *C. papaveracea* (see mating type gene results below).

Mating type gene distribution in Crivellia

The mating type screening of the 22 *C. papaveracea* and *B. papaveris* isolates with *MAT1-1* and *MAT1-2* specific primers showed the respective characteristic band of ca. 300 and 220 bp and divided the isolates into three groups. Three *C. papaveracea* isolates had *MAT1-1* only, 11 *C. papaveracea* isolates *MAT1-2* only, and all eight *B. papaveris* isolates had both mating type diagnostic PCR fragments (Fig. 86).

Arrangement of MAT *genes in* Crivellia

To investigate *MAT* locus architecture in *C. papaveracea* and *B. papaveris*, partial DNA sequences of the *MAT* loci were generated for each of the three *MAT* groups obtained by PCR screening. Sequencing coverage obtained for *C. papaveracea* isolate P128 (*MAT1-1*), *C. papaveracea* isolate P396 (*MAT1-2*), and *B. papaveris* isolate P390 (*MAT1-1, MAT1-2*) is illustrated in Fig. 87. DNA sequences were submitted to GenBank as AY377898, AY380313, and AY382474. The partial mating type region sequences obtained from *C. papaveracea* isolates P128 and P396 demonstrated that the 5′ end of the mating type genes lay downstream of an ORF1 of unknown function (Fig. 87), just as in the closely related heterothallic species *A. alternata* and *C. heterostrophus*. The partial mating type region fragment of the homothallic *B. papaveris* isolate P390 contained both *MAT1-2* and *MAT1-1* genes. The arrangement of the mating type genes in *B. papaveris* isolate P390 is most similar to *C. homomorphus*, where *MAT1-2* and *MAT1-1* are fused into a single ORF (Yun et al. 1999). The identity of the *Crivellia MAT* sequences obtained was confirmed by BLAST searches at GenBank (Altschul et al. 1997) which returned *MAT* regions of *A. alternata* and *C. heterostrophus* as closest matches (data not shown).

Delimitation of Crivellia *idiomorphs*

The lengths of the idiomorphs differ in ascomycetes (Bennett et al. 2003). DNA sequence comparisons between *Crivellia MAT* regions showed that the idiomorph 5′ end extended 90 bp into ORF1 (data not shown). This was comparable to *Stemphylium* (Inderbitzin 2004), whereas in *A. alternata* the idiomorph terminated 4 bp short of reaching ORF1. The 3′ idiomorph end was not sequenced.

Evidence for extant outcrossing in Crivellia papaveracea

Mating type genes of a sexual heterothallic are expected to segregate in a one-to-one ratio. We randomly picked 14 ascospores from the content of one ascoma of *C. papaveracea* isolate P354, and used PCR to screen for the presence of mating type (strains used for screening are not contained in Table 1). Twelve strains had the *MAT1-1* specific band, two the *MAT1-2* specific band, none had both. Finding both mating types from one fruitbody is evidence for meiotic segregation and implies extant outcrossing in *C. papaveracea*. Lab crosses were set up between *MAT1-1* and *MAT1-2* strains, but no evidence for sexual reproduction was observed after several months.

Discussion

The new genus *Crivellia*

The morphological differences between *Pleospora*/*Stemphylium* and *Crivellia* are reflected in the results of phylogenetic analyses: Both 18S and ITS analyses placed *Crivellia* with *Alternaria* rather than *Pleospora*. *Crivellia* appeared with *Alternaria alternata* as its closest relative in 18S analyses (Fig. 83), and based on ITS within the *Alternaria* group of fungi (Fig. 84). High bootstrap per-

centages and Shimodaira–Hasegawa test results supported the separation of *Crivellia* from *Pleospora*. *Crivellia* shares with *Alternaria* and *Ulocladium* of the *Alternaria* group the formation of asexual spores in chains that are extended apically. *Ulocladium* has obovoid asexual spores, those of *Alternaria* are ovoid (Simmons 1967), whereas in *Crivellia* asexual spores are cylindrical. However, Pryor and Gilbertson (2000) showed that *Ulocladium* is not monophyletic, and thus the shape of asexual spores does not always reflect phylogenetic relationship.

Phylogenetic analyses show that the *Alternaria* group contains three teleomorphic genera, *Lewia*, *Allewia*, and *Macrospora*. Together with the anamorphic *Alternaria* and *Ulocladium*, these form a monophyletic group (Pryor and Bigelow 2003) that also includes *Crivellia* (Fig. 84). The closest sexual relative of *Crivellia* within the *Alternaria* group remains uncertain. In phylogenetic analyses of the ITS, *Crivellia* clustered, with low support, with three species without known sexual states: *A. brassicicola*, *A. japonica*, and *Ulocladium alternariae* (Fig. 84).

An alternative to erecting the new genus *Crivellia* would have been the inclusion of *Crivellia* in *Lewia, Allewia*, or *Macrospora*. However, molecular phylogenetic evidence does not resolve any close relationship between *Crivellia* and a teleomorphic genus. We consider establishment of *Crivellia* to be the most appropriate option given morphological characters (explained below) that precluded placing *Crivellia* species in any of the earlier defined teleomorphic genera in the *Alternaria* group.

Lewia Barr & Simmons is typified by *L. scrophulariae* (Desm.) Barr & Simmons and has an anamorph, *Alternaria conjuncta* Simmons. Simmons (1986) included five species of *Lewia* each with distinctive *Alternaria* anamorphs. The ascospores of *Lewia* have 5–6(–7) transverse septa unlike the transversely three-septate ascospores of *Crivellia papaveracea* (Figs. 1, 2, 28–30). The *Alternaria* anamorphs have conidia that are dictyosporous, catenate, and strongly narrowed towards the apex unlike the phragmosporous conidia of *C. papaveracea* and *B. papaveris* (Figs. 7, 31–37, 43–54, 56–68, 80–82).

Allewia Simmons (1990) is typified by *A. proteae* Simmons, which has an anamorph *Embellisia proteae* Simmons. Simmons (1990) included two species of *Allewia* each with distinctive *Embellisia* anamorphs and treated three new species of *Embellisia* plus *E. dennisii* (M.B. Ellis) Simmons, a transfer from *Alternaria*. The ascospores of *Allewia* have 7(–9) transverse septa unlike the transversely three-septate ascospores of *Crivellia papaveracea* (Figs. 1, 2, 28–30). The *Embellisia* anamorphs are dictyosporous with very prominent transverse septa unlike the phragmosporous conidia of *C. papaveracea* and *B. papaveris* (Figs. 7, 31–37, 43–54, 56–68, 80–82).

Macrospora Fuckel based on *M. scirpicola* (DC.: Fr.) Fuckel has applanodictyoascospores and based on the nature of the disc-like opening of the ascoma, was placed in Diademaceae (Shoemaker and Babcock 1992). The ascospores of *Crivellia* are terete. *Nimbya scirpicola* (Fuckel) Simmons (1989) is the anamorph of *M. scirpicola*. The *N. scirpicola* conidia are long and tapered to the apex, with a truncate prominent scar, characters not shared by conidia of *Crivellia papaveracea* or *B. papaveris* (Figs. 7, 31–37, 43–54, 56–68, 80–82).

Crivellia contains at least two species

Phylogenetic and morphological species were congruent in *Crivellia*. Two phylogenetic species were recognized. The first consisted of isolates with identical DNA sequences at all loci, and corresponded to the morphological species *B. papaveris*. The second was more variable, and consisted of strains of *C. papaveracea* (Fig. 85). The presence of two species in the *Crivellia* clade was first noted by Neergaard (1940, 1941). He compared his collections to the type material of the asexual state of *C. papaveracea*, and differentiated between poppy species infected by *C. papaveracea* or *B. papaveris*. We did not study his specimens and cannot be certain that his two species corresponded to ours. Meffert (1950) also differentiated two taxa in the *Crivellia* clade. We studied two isolates representative of her two varieties, and found that her strains (P411 and P412, Table 1) corresponded, respectively, to *B. papaveris* and *C. papaveracea*. More recently, using AFLP and isolates of which five were also included in this study (Table 1), Farr et al. (2000) found two groups corresponding to *C. papaveracea* and *B. papaveris*.

There might be additional species in *Crivellia*, as for example Zogg (1945) described an opium poppy fungus similar in morphology to the two species of *Crivellia* (Meffert 1950). On *Papaver somniferum*, Zogg's (1945) fungus formed the sexual state and macroconidiophores during winter, and microconidiophores in the summer. In culture, from all stages microconidiophores were obtained. He did not mention miscrosclerotia or investigate fruitbody formation in culture, important characters that distinguish the two *Crivellia* species described here. No cultures from Zogg's study were available for inclusion in our studies.

Characterization of the two *Crivellia* species

Crivellia papaveracea and *Brachycladium papaveris* are morphologically similar, but distinct. Based on the 22 isolates examined in this study, only *C. papaveracea* forms microsclerotia and macroconidiophores, whereas chlamydospores and hyphal cells excreting a pigmented substance are confined to *B. papaveris*.

Other characters, including conidium size and septation or physiology and perhaps levels of virulence may distinguish the two species. Meffert (1950) and Farr et al. (2000) agreed that while the conidia of *C. papaveracea* and *B. papaveris* overlapped in size and in numbers of septa, the *B. papaveris* conidia tended to be larger, with more septa. Meffert (1950) found that 99% of *C. papaveracea* conidia had four or fewer septa, whereas in *B. papaveris* this number was 76%, with some up to 12 septate conidia. However, in both fungi three-septate conidia were most abundant, accounting for 54% and 43% of all conidia, respectively. Both Meffert (1950) and Farr et al. (2000) measured hundreds of conidia to arrive at their conclusions. We found that without this considerable effort it was impossible to differentiate between *C. papaveracea and B. papaveris* conidia, and judged these characters to be impractical for efficient species delimitation.

Farr et al. (2000) found differences in growth rates of

C. papaveracea strain P350 and *B. papaveris* strain P351 (Table 1) under various temperature and nutrient regimes. If confirmed with more isolates and appropriate statistical analyses, these growth differences may be useful for species delimitation and recognition. Meffert (1950) found that *C. papaveracea* strain P412 could reduce malachite green while *B. papaveris* strain P411 could not (Table 1). This potentially diagnostic character should be investigated with a greater number of isolates. Several authors agreed that *C. papaveracea* and *B. papaveris* were both pathogenic on opium poppy, displaying similar symptomology (Bailey et al. 2000). Whereas Meffert (1950) did not detect any differences in virulence between *C. papaveracea* strain P412 and *B. papaveris* strain P411 (Table 1), *B. papaveris* strain P351 seemed to form more appressoria than *C. papaveracea* strain P350, and the former was also more virulent and competitive on opium poppy than the latter (Bailey et al. 2000; O'Neill et al. 2000). These results should be tested with additional strains before they are postulated as interspecific differences of *C. papaveracea* and *B. papaveris*.

Mating system

Mating system differs between species of *Crivellia*. Farr et al. (2000) found that *B. papaveris* was homothallic. *Crivellia papaveracea* was not observed to form the sexual state in culture. However, cultures from single ascospores of *C. papaveracea* from a single field-collected ascoma carried either *MAT1-1* or *MAT1-2*, as expected in a heterothallic fungus following meiotic segregation of two alleles. Sexual recombination was also supported by the permutation tail probability test of the ITS, *EF*, and *GPD* datasets which failed to reject the null hypothesis of panmixia in *C. papaveracea*. No such tests were possible in *B. papaveris* since no molecular variation was observed in the eight isolates examined. Further evidence of a difference in mating system between *C. papaveracea* and *B. papaveris* was from analyses of the *MAT* locus which has been thoroughly investigated in the closely related genus *Cochliobolus* (Yun et al. 1999).

As in *Crivellia, Cochliobolus* contained both selfing and outcrossing species. In *Cochliobolus* selfing species were derived from outcrossers, and selfing species differed from outcrossers at the compatibility determining *MAT* locus. Outcrossers either had the *MAT1-1* or *MAT1-2* allele at the *MAT* locus, whereas some selfing species had both alleles fused end to end at the *MAT* locus (Yun et al. 1999). As in *Cochliobolus*, isolates of the outcrossing *C. papaveracea* either had one or the other allele at the *MAT* locus, whereas all strains of the selfing *B. papaveris* had both alleles at the *MAT* locus (Fig. 86), arranged in an unique way. The *MAT1-1* allele was fused to the 3′ end of a *MAT1-2* gene, 43 bp upstream of the *MAT1-2* 3′ end (Fig. 87). Otherwise, reflecting close phylogenetic relationship, the *MAT* locus upstream gene in *Crivellia* was ORF1, as in *Cochliobolus* and *A. alternata*.

Thus, mating system, and *MAT* gene arrangement in particular, was a useful distinguishing character for the isolates of *C. papaveracea* and *B. papaveris* used in this study.

The sexual state of *Brachycladium papaveris*

Farr et al. (2000) were able to obtain the sexual state of *B. papaveris* strain P351 in the lab on inoculated leaves of *P. somniferum*. Since this material has not been preserved, we were unable to describe the *Crivellia*-state of *B. papaveris*. That *B. papaveris* does have a sexual state is also supported by observations made by Meffert (1950) and possibly Schmiedeknecht (1962) who reported a method to induce fruitbody formation by a homothallic opium poppy pathogenic fungus, possibly *B. papaveris.* In absence of any natural collections of the sexual state of *B. papaveris*, Schmiedeknecht's method might be useful in generating material of the *B. papaveris* sexual state.

Evolutionary origin of the self-compatible *Brachycladium papaveris*

In the ascomycete genera *Cochliobolus* and *Stemphylium* homothallism originated from heterothallism, in some cases possibly by a crossover leading to fused *MAT* loci conferring self-compatibility (Inderbitzin et al. 2005; Yun et al. 1999). In *Crivellia*, a crossover between outcrossing ancestors might also have resulted in the fused *MAT* loci found in the homothallic species (Fig. 87). Yun et al. (1999) compared mating type genes of closely related heterothallic ancestors and found that a crossover probably gave rise to the *MAT* gene fusion in *Cochliobolus homomorphus*. Unlike *C. homomorphus*, the *MAT* genes in *B. papaveris* isolate P390 were not fused into a single ORF, but *MAT1-2* was lacking 43 bp at the 3′ end, and was fused to a ORF1-*MAT1-1* spacer region (Fig. 87). A 4 bp motif present at the fusion junction between *MAT1-2* and *MAT1-1* in *B. papaveris* isolate P390 was shared with homologous regions of the *MAT1-1* spacer region in *C. papaveracea* strain P128 and *MAT1-2* gene in A. *alternata* representing a potential crossover site (Fig. 88). The motif present at the time of crossover might have been shortened by post-crossover mutations, as well as by the use in these comparisons of the relatively distant *A. alternata MAT1-2* gene as hypothetical ancestor. However, in *Saccharomyces cerevisiae* a stretch of four shared nucleotides is sufficient for a crossover to occur (Schiestl and Petes 1991).

It is further possible that the crossover resulted in reproductive isolation between self-compatible and self-fertile isolates, and thus to the creation of the two extant species of *Crivellia*.

Conclusions

The primary goal of this study was to lay the basis for a stable taxonomic framework and means of identification for the benefits of further work in this group of fungi. We have shown that *Crivellia papaveracea* is a heterothallic species in the *Alternaria* group, and that *Brachycladium penicillatum* is the correct name for its asexual state. We show that *B. penicillatum* can be distinguished from the homothallic species *B. papaveris* based on molecular and morphological characters. From a taxonomic point of view, future work required is the description of the sexual state of *B. papaveris*, as well as the examination of a larger number of isolates. More extensive analysis of this group of fungi has the potential to yield additional species and diagnostic characters useful for their identification.

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